INDUCTION OF ERYTHROID DIFFERENTIATION IN RAT ERYTHROLEUKAEMIC (REL) CELLS

by

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ABSTRACT

The work presented here was an attempt to characterise some properties of a rat erythroleukaemic cell line (REL), in particular, the ability of these cells to undergo erythroid differentiation following exposure to dimethylsulphoxide (DMSO).

Erythroid differentiation was readily demonstrated by the appearance of haemoglobin in REL cells, using the benzidine reaction. Although it required some 72 hours exposure to DMSO before significant numbers of haemoglobinized cells could be detected, it appeared that some cells became committed to differentiate after as little as 8 hours exposure. It was possible to propose a sequence of events when REL cells were exposed to DMSO. After 4 hours of DMSO, DNA synthesis was down-regulated and REL cells started to shift out of S-phase of the cell cycle (i.e. proliferative) and started to accumulate in G_0/G_1 (i.e. quiescent). This loss of proliferative ability was reflected in a reduction in cloning efficiency, i.e. some cells could no longer divide. Cells became committed to differentiation by 8 hours and by 24 hours, gross morphological changes could be observed, e.g. a significant reduction in cell size, cells adopting a smooth surface appearance as demonstrated by scanning electron microscopy (SEM). Cells producing haemoglobin could be detected by 72 hours of DMSO and the number of cells rose to a maximum after about 96 hours. It would appear that haemoglobin production is a relatively late event in DMSO-induced erythroid differentiation of REL cells.

To try to identify the biological activities that regulate differentiation, REL cells were adapted to a chemically defined, serum-free (SF) medium. Although the growth and division of these cells was not significantly different from cells grown in serum-containing medium, without serum, REL cells could not be induced to differentiate. Even when SF medium was supplemented with combinations of known growth factors / cytokines, no significant erythroid differentiation was obtained.

It is because of this ability to differentiate along an erythroid pathway that the REL cells are regarded as erythroleukaemic. However, under certain culture

conditions, REL cells developed characteristics associated with monocyte / macrophage maturation as demonstrated by morphology, appearance under SEM, cytochemical analyses, and expression of a macrophage-associated surface antigen. It would appear that REL cells are not restricted to the erythroid lineage, but, in fact, they retain the potential to mature along additional lineages.

Brief cytogenetic analysis revealed the presence of a chromosomal abberation, namely, the translocation of the major portion of chromosome 3 to the terminal region of chromosome 1. The area of the breakpoint on chromosome 3 contains the proto-oncogene, c-abl. It may be that this translocation subverts the normal expression of c-abl and this aberrant expression contributes to the unregulated growth of REL cells.

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SECTION 1

INTRODUCTION

1.1 Historical Perspective

The vital importance of blood was probably recognised from earliest times. Certainly, in the Old Testament "to shed blood" meant "to kill", i.e. if enough blood is lost, life ceases. Although blood was recognised to be essential for life, its nature, by necessity, remained obscure until relatively recently. It was not until the advent of the microscope that a means became available to study the nature of blood. One of the most skilful of the early microscopists was Antonj van Leeuwenhoek (1632-1723) of Delft, Holland. It is generally Leeuwenhoek who is credited with first describing the red blood cell (1) although his contemporaries, Jan Swammerdam (1637-1680) and Marcello Malpighi (1628-1694) also recorded their observations on the cellular nature of blood. Some hundred years later, William Hewson (1739-1774) reported the presence of the relatively much less numerous "colourless" blood cells, i.e. white blood cells (2).

From Leeuwenhoek's first letter to the Royal Society in April 1673, it was nearly two hundred years (i.e. 1868) before the origins of the blood cells were first recognised. Ernst Neumann (1834-1918) (3,4) and, almost simultaneously, Giulio Bizzozero (1849-1901) (3,4) demonstrated that, in the adult organism, the main site of red blood cell formation is the bone marrow. Subsequently, they both reported that, in the case of adult humans, red blood cell production is limited to the bone marrow (3). It is interesting to note that these earlier reports were highly controversial and evoked great scepticism amongst many of the contemporaries of Neumann and Bizzozero. It was not until the end of the century, a period of some twenty odd years, that the bone marrow was universally accepted to be the site of haematopoiesis in adult man.

It is worth mentioning that up until this time there was a general assumption that blood cells, once formed in the embryo, persisted in the body throughout life. However, it was Neumann who recognised that this was not so (3), that in fact blood formation was a continuous process occurring during post-natal life.

It was Artur Pappenheim (1870-1916) who first proposed that all the different types of blood cells arose from one common ancestor. Using staining methods, he noted that various transition forms of cells could be traced back to a relatively featureless type of mononuclear cell, which he termed the "lymphoidocyte"(4). From a morphological point of view this cell appeared so primitive that he considered it to be the common ancestor of all blood cells. This monophyletic theory of Pappenheim's was based solely on morphological criteria. The severe limitations of this morphological analysis could not be overcome until some new experimental tools became available, viz, radiation biology.

The first step forward came in Chicago where Jacobson and co-workers demonstrated that mice given otherwise lethal whole-body irradiation could be protected by partial shielding of haematopoietic tissue -they exteriorized the spleen and excluded it from the radiation (5,6). Although there was some controversy over whether this haematological recovery was due to a humoral factor or due to living intact cells, the matter was resolved in 1956 in Harwell by Ford and co-workers who used a chromosome marker to clearly demonstrate that this radioprotective effect was cellular, i.e. intact viable cells in the graft were responsible for the restoration of the haematopoietic tissue (7). This was amply confirmed by several other groups working independently (8, 9, 10).

The watershed came in 1961 when Till and McCulloch, working in Toronto, reported on the radiation sensitivity of normal mouse bone marrow cells (11). They reported an assay in which mice were lethally irradiated and then injected with low numbers of bone marrow cells (10^4-10^5). This low number of cells is not sufficient to ensure long-term survival of the recipient. However, when the surviving animals were sacrificed after 10 or 11 days, discrete macroscopic nodules were clearly visible

on the surface of their spleens. Histologically, each nodule consisted largely of proliferating recognisable haematopoietic cells and their precursors. In addition, the number of spleen colonies was found to be directly proportional to the number of marrow cells injected, and granulopoiesis, erythropoiesis and megakaryocytopoiesis could be observed in the nodules. Their results were compatible with the assumption that single viable cells from the marrow graft were able to give rise to colonies in the spleen. However, because of their uncertainty over the identity of the cells, they proposed the term "Colony-Forming Unit" (CFU) to designate the cell (or cells) which gave rise to a single colony.

The clonality of these spleen colonies was soon demonstrated by more direct evidence from several groups working independently. Becker et al used marrow cells that had been irradiated to produce chromosomal aberrations (12). Eleven days after these cells were injected into irradiated mice, individual spleen colonies were removed and subjected to chromosomal analysis. This analysis showed that the same chromosomal markers were present in 95-99% of the cells of colonies that had chromosomal markers. Since the chromosomal aberration was produced by a random process, it was highly improbable that an identical abnormality should occur in each cell and this provided good evidence for the clonal origin of the splenic nodules. Fowler et al examined 14-day colonies and found that 83%-98% of the cells from each marked colony contained the characteristic marker chromosome of that colony (13). In addition, Welshons transplanted a mixed suspension of marrow cells from normal and chromosomally marked donor mice and found that none of the spleen nodules in the host had a mixed karyotype (14).The only explanation for these observations was that the original cell carrying the marker was the parent of all the other cells in that colony. Furthermore, these colonies could consist purely of one cell lineage (erythroid, granulocytic or megakaryocytic) or a mixture of two, or even all three of them (13, 15,16).

By now, compelling evidence had accumulated for the concept that there was a pluripotential stem cell that could give rise to all the morphologically identifiable

cells of the blood and bone marrow. However, the nature of this relationship remained to be elucidated. The picture began to take shape with the introduction of techniques that allowed the in vitro culture of bone marrow cells.

1.2 In Vitro Culture of Bone Marrow

The earliest report of the in vitro culture of bone marrow cells appears to be the work of Carrel and Burrows in 1910 (17). The cells were cultured over a relatively short term of between 6 and 24 hours. Some years later, Osgood and Muscovitz introduced a rather complicated cell suspension method for culturing bone marrow for haematologic and metabolic studies (18).

However, the modern era of bone marrow culture dawned some thirty-odd years ago when two reports appeared in quick succession describing the in vitro culture of mouse haematopoietic cells. In Israel, Pluznik and Sachs cultured adult mouse spleen cells on a feeder layer of mouse embryo cells. The concluded (mistakenly) that the colonies formed were composed of "mast" cells (19). Shortly after this report, Bradley and Metcalf described their technique for the growth of colonies from single cell suspension of mouse bone marrow (20). They correctly identified the nature of the cells that made up the colonies, viz, neutrophilic granulocytes and macrophages. Pluznik and Sachs subsequently showed that the cells of the feeder layer could be replaced by "conditioned medium" (21). This consisted of mouse embryo cells sustained in tissue culture medium for 7 days. The resulting supernatant contained the factors necessary for spleen cell growth. The term "Colony Stimulating Activity" (CSA) was coined to denote the factor or factors present in conditioned medium, or secreted by feeder cells, which stimulated the proliferation in vitro of granulocytes/macrophages.

The clonal nature of the colonies formed was implied by the demonstration of a linear relationship between the number of cells plated and the number of colonies grown. This clonality was later confirmed by colony mapping and single cell cloning experiments (22). The cell of origin of these colonies was

termed the "colony forming unit in culture" (CFU-C) as opposed to the previously discussed colony forming unit in spleen (CFU-S). In the fulness of time this feeder layer technique was adapted to allow the growth of human bone marrow cells (23).

With the demonstration that colonies of granulocytes and/or macrophages could be grown in semi-solid medium Axelrad and colleagues, working in Toronto, succeeded in growing erythroid colonies from mouse foetal liver cells (24). They argued that the rather vague term, CFU-C, should be further dissected to CFU-G for granulocytic colonies and CFU-E for these erythrocytic colonies. The clonal nature of these erythroid colonies was demonstrated by direct observation using time-lapse cinemicrography (25).

It became clear that this CFU-E was a relatively mature cell (26). Subsequently, it was shown that when the erythropoietin concentration was increased and the period of incubation prolonged, more immature cells could be grown which produced small colonies which were not distributed homogeneously in the cultures but rather occurred in bursts (27). The previous operational nomenclature was again used, with a slight modification, and the cell that gave rise to an erythroid burst was called a "burst forming unit-erythroid" (BFU-E)

Reports of additional colony-forming cells continued to emerge. Eosinophils were successfully cultured from both human peripheral blood cells and bone marrow cells (28,29). Metcalf et al reported a technique for growing megakaryocytes from mouse bone marrow (30). A few years later, human megakaryocyte colonies were grown from both peripheral blood cells and bone marrow cells (31,32).

The colony-forming cells so far described are "committed" in that they are restricted in their development to only one cell lineage (or two in the case of the granulocyte / macrophage progenitor cell). Subsequently, an assay became available for a multipotential haematopoietic progenitor in both the mouse and the human system (33,34). The haematopoietic colonies generated in these cultures contained terminally differentiated cells of several lineages,e.g.granulocyte/macrophage,

erythrocyte and megakaryocyte.This progenitor cell is variously known as the "CFC-Mix" (Colony-Forming Cell-Mixed), "Multi-CFC" (Multipotential Colony-Forming Cell) or"CFU-GEMM" (Colony-FormingUnit-Granulocyte,Erythrocyte, Macrophage, Megakaryocyte).

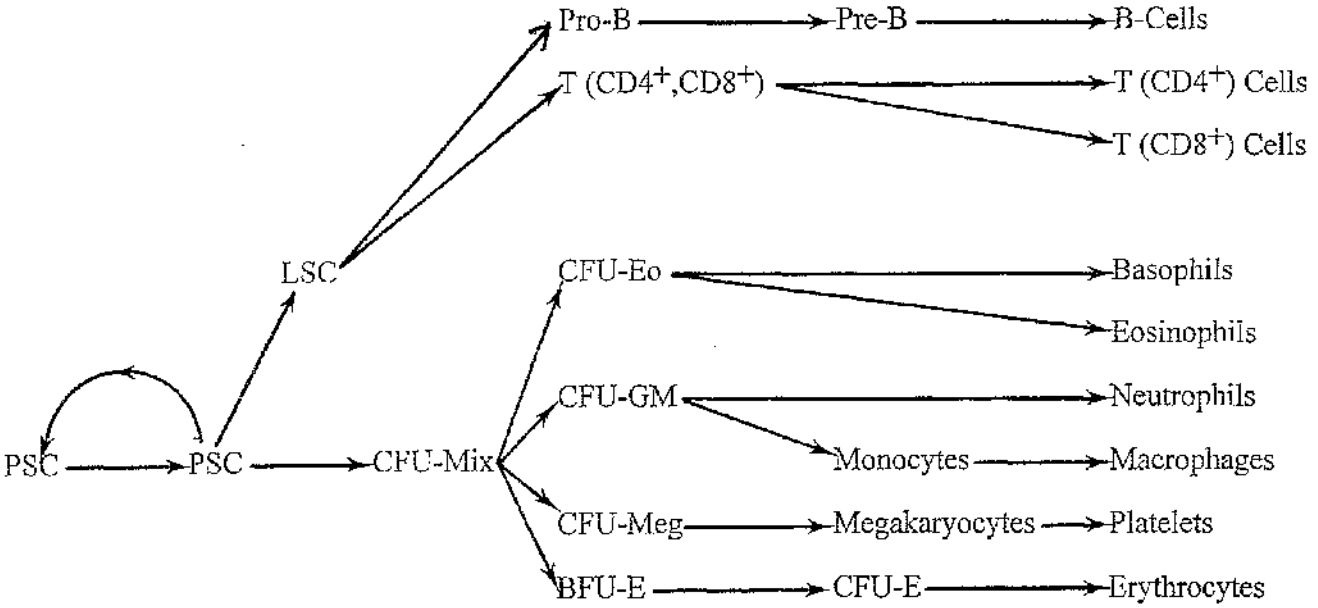
For the sake of accuracy it should be kept in mind that the terms "haematopoietic stem cell" and "CFU-S" are not, strictly speaking, synonymous.Although some CFU-S are stem cells, as demonstrated by their pluripotential nature and self-renewal ability (35), there is evidence to indicate that CFU-S represent a heterogeneous population of cells differing in their ability to self-renew (36). It would appear that there is a primitive stem cell which gives rise to CFU-S (37). In support of this, Hodgson and Bradley proposed a class of primitive haematopoietic stem cell, "pre-CFU-S", which accumulates in the bone marrow to give rise to cells capable of growing in the spleen (38).

Based on the colony assays it is convenient to represent the hierarchial nature of haematopoiesis in the form of a lineage diagram (Fig 1). Lest this representation be misleading, it should be remembered that considerable amplification takes place within the differentiating cell populations. For example, if a particular stem cell is committed to differentiate along the erythroid pathway, i.e. BFU-E, it retains considerable growth capacity, i.e. it may divide several times as a BFU-E. Subsequently, when these cells have matured to morphologically recognisable cells, e.g. normoblasts, they still possess considerable proliferative ability (39). This amplification can be represented diagrammatically (Fig 2).

1.3 Regulation of Haematopoiesis

From the earliest experiments where murine haematopoietic cells were successfully cultured in vitro, it was obvious that proliferation and colony formation required the continued presence of factors elaborated from the cells of the feeder layers (19,20). Subsequent work showed that the feeder cells need not be capable of proliferation and indeed could be replaced by medium harvested from

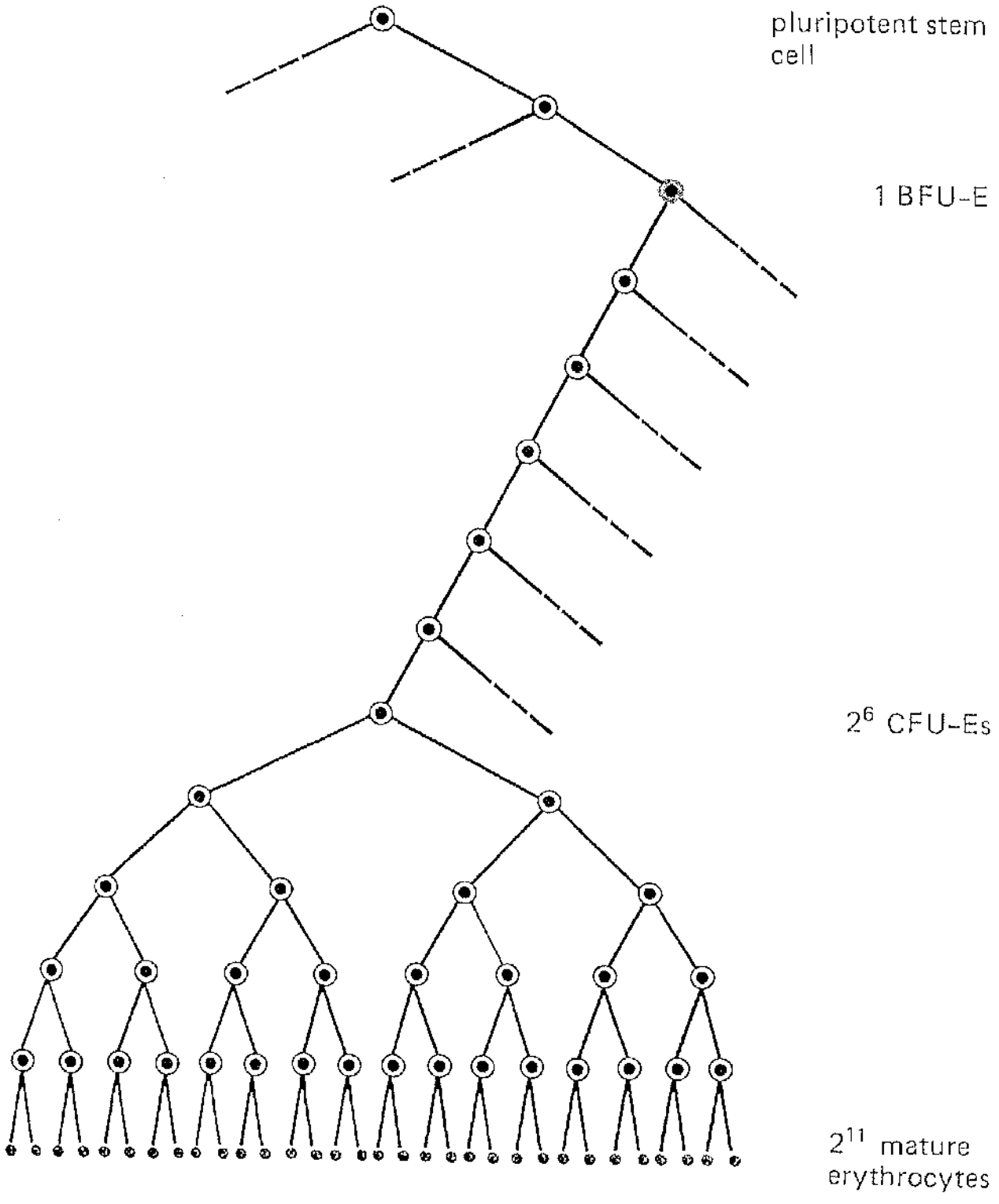
Figure 1 **Scheme of haematopoiesis.**



Abbreviations : PSC = pluripotent stem cell; LSC = lymphoid stem cell; CFU-Mix = colony forming unit-mixed; CFU-Eo = colony forming unit-eosinophil; CFU-GM = colony forming unit-granulocyte-macrophage; CFU-Meg = colony forming unit-megakaryocyte; BFU-E = burst forming unit- erythroid; CFU-E = colony forming unit-erythroid.

Figure 2

Amplification within the haematopoietic system.



liquid cultures of feeder cells, i.e. conditioned medium (21). Other studies showed that small volumes of mouse serum (40), human serum (41), or human urine (42) could be incorporated into the agar medium and serve as a suitable stimulus for colony formation in vitro by haematopoietic cells.

Various descriptive terms were proposed to describe this activity:- "colony stimulating factor (CSF)" (43), "colony stimulating activity" (CSA) (44), "inducer of colony formation" (45), "conditioning factor activity" (46), "macrophage and granulocyte inducer" (MGI) (47). By convention, the term "CSF" was most commonly used to describe this stimulatory activity. The tissues that produced CSF were many and varied:-spleen (48), embyro kidney (49), vascular cells (50), lung (51), placenta (52). In addition, a variety of human cell lines have been established which elaborate CSF, e.g. the T-lymphoblast cell line (Mo) which was derived from a patient with a T-cell variant of hairy-cell leukaemia (53), the monocyte-like cell line GCT which was isolated from a lung metastasis of a fibrous histiocytoma (54), a pancreatic carcinoma cell line (55), a cervical carcinoma cell line (56), and T-cell lines from normal individuals (57) or leukaemia-lymphoma patients (58). This apparent ubiquity of sources of CSF has lead to speculation that maybe all tissues in fact produce varying amounts of CSF, some more easily detectable than others (59).

Interestingly, CSF levels were elevated in tissues and serum in situations where increased production of granulocytes and monocytes would be required, e.g. during infections (60,61,62,63) or after injection of bacterial products (64).This seemed to indicate that CSF was indeed a genuine regulator *in vivo* of granulocyte - macrophage populations and therefore its characterisation became a priority.

It soon became apparent that CSF was not a single entity, and, in fact, there appeared to be several distinct CSF's with overlapping actions on granulocyte-macrophage populations.

By 1975, the first CSF had been purified, in this instance from human urine (65). It should be remembered that these activities were originally identified through colony formation in in-vitro culture systems. This is reflected in the

Characteristics of Human Haematopoietic Growth Factors

Growth Factor	Molecular Mass (kd)	Chromosomal Location of Gene	Cellular Source	Progenitor Cell Targets
G-CSF	18-22	17q11.2-21	Monocytes Fibroblasts Endothelial cells	CFU-G
M-CSF	70-90	5q33.1	Monocytes Fibroblasts Endothelial cells	CFU-M
GM-CSF	14-35	5q23-31	Monocytes Fibroblasts Endothelial cells T-lymphocytes	CFU-Mix, CFU-GM, CFU-G, CFU-M, CFU-Eo, CFU-Meg, BFU-E.
IL-3	14-28	5q23-31	T-lymphocytes	CFU-Mix, CFU-GM, CFU-G, CFU-M, CFU-Eo, CFU-Meg, BFU-E.
Erythropoietin	34-39	7q11-22	Kupffer cells Peritubular cells of the kidney	CFU-E, late BFU-E, CFU-Meg

nomenclature used to describe individual CSF's, i.e. a prefix is used to indicate the major cell populations produced as a consequence of the action of a particular CSF.This CSF isolated by Metcalf's group stimulated macrophage colony formation in cultures of mouse bone marrow cells.Although originally known by the trivial name of "urinary CSF", it is now properly referred to as "macrophage - colony stimulating factor" (M-CSF), i.e. a CSF that preferentially stimulates the formation of colonies of macrophage cells.

Conditioned media provided rich sources of CSF's, allowing the purification of murine (66) and human (67) granulocyte-macrophage colony stimulating factor (GM-CSF), murine (68) and human (69) granulocyte colony stimulating factor (G-CSF). The fourth and final CSF that was isolated was found to be less restrictive in its activity, possessing the ability to stimulate the formation of megakaryocytes (70) erythroid and multipotential colonies in cultures of murine bone marrow (71). This activity was subsequently purified by Metcalf's group (72) and given the name multipotential CSF (Multi-CSF). However, this activity had already been isolated (73). Ihle et al had identified an activity in WEHI-3 conditioned medium that induced the enzyme 20 α-hydroxysteroid dehydrogenase in murine splenic lymphocytes. They had named this molecule interleukin-3 (IL-3). Multi-CSF and IL-3 are now accepted as synonyms for the one activity.

The problem which arose now was that, although all 4 CSF's had been purified, the amounts of starting materials available were far too small to permit even the simplest clinical study thus making it impossible to determine if the CSF's did indeed possess any regulatory function in vivo.

All this changed with the application of molecular biological techniques. Within a 3 year period (1984-7) complementary DNA's (cDNA's) were cloned for each of the 4 murine and human CSF's (128). The characteristics of the human haematopoietic growth factors are summarised in Table 1. (74).

These colony stimulating factors are the classic haematopoietic growth factors. However, there are other, many and varied, molecules which have a

regulatory function in haematopoiesis. The most obvious one is erythropoietin (Epo) which has its predominant effect on committed erythroid cells, promoting their proliferation and differentiation into proerythroblasts (75). Its importance in regulating red cell production in vivo has been clearly established (76).

A major group of regulatory molecules is the ever-increasing"Interleukins" (IL's), presently numbering fifteen (IL-15) (77). Some of these interleukins have their primary effect on the development of lymphoid cells. For example, IL-2 is a T-cell derived cytokine which stimulates growth and differentiation of T cells, B cells, Natural Killer (NK) cells and Lymphokine Activated Killer (LAK) cells (78). IL-7 is a growth factor for progenitor B cells and T cells (79).

Other interleukins can influence myelopoiesis. IL-5 is somewhat restricted in its activity, being a powerful stimulus for the proliferation of eosinophils (80). IL-1 has a number of effects on a variety of cell types. Within the haematopoietic system, it can act either directly on early stem / progenitor cells to prolong their survival (81) or indirectly by influencing the release of CSF's from accessory cells (82, 83). IL-3 stimulates growth and differentiation of a broad spectrum of haematopoietic cell lineages. It supports growth of multilineage colonies (84, 85) as well as all the committed progenitors (86). However, many of the activities of IL-3 are enhanced or depend upon co-stimulation with other cytokines. For example, IL-6 has activity on megakaryocytes and platelets (87, 88) especially in combination with IL-3 (89). Similarly, IL-11 can synergize with IL-3 to stimulate haematopoiesis in vitro (90).

Many other activites have been reported, notably Stem Cell Factor (SCF) (also known as kit ligand (KL)), Transforming Growth Factor-beta (TGF-β), Macrophage Inflammatory Protein-1 alpha (MIP-1α), and Leukaemia Inhibitory Factor (LIF). SCF has a wide range of activities and appears to act on early myeloid cells where it can have a potent synergistic effect in conjunction with other growth factors (91). TGF-β is considered to be a negative regulator and can down-modulate the expression of receptors for G-CSF, GM-CSF, and IL-3 (92). In addition, TGF-β appears to maintain early progenitors in a non-proliferative quiescent state, thereby preventing them

entering the cell cycle (93). MIP-1α can also have a suppressive activity on early haematopoietic cells (94) and can be myeloprotective (95). In combination with IL-3, LIF can potentiate the stimulation of megakaryocyte precursors (96). It also has a somewhat unique effect on embryonic stem (ES) cells. ES cells exhibit a predisposition in culture to differentiation commitment with loss of totipotentiality. However, incubation with LIF prevents this differentiation commitment and allows the cells to retain their totipotentiality (97).

This interacting network of various regulatory molecules is a feature of haematopoiesis, and synergy is often observed using a wide range of growth factor combinations (98). The fine control of haematopoiesis is a highly complex phenomenon requiring a subtle balance between stimulatory activities (like the CSF's) and suppressive influences (like MIP-1α and TGF-β).

For all practical purposes, the stem cells are immortal (99) in that they persist throughout the lifespan of the organism. In fact, there is a vast proliferative reserve built into the system that would be sufficient to last several times the normal lifespan. This is elegantly demonstrated in transplantation studies where relatively few donor cells are required to reconstitute fully the haematopoietic system of the recipient (100). For example, infusion of as few as 10^4-10^5 marrow cells into an irradiated mouse is sufficient to restore haematopoiesis. Since a normal mouse has about 3×10^8 marrow cells, the infused amount represents only 0.01% of this total. Furthermore, these transplant recipients have a normal lifespan and their haematopoietic system shows no evidence of decline with age (101). In addition, it has been amply demonstrated in murine studies that bone marrow cells from a transplant recipient can themselves be used successfully in further transplantation procedures (102), although with repeated serial transplantation, the ability of the donor marrow to reconstitute the recipient mice is eventually lost (103, 104, 105).

The scale of this process is quite remarkable when one considers that every day some 2×10^{11} erythrocytes, 1×10^{10} granulocytes and 4×10^{11} platelets enter the circulation, in addition to lymphocytes and monocytes (3). With a production

system of this magnitude, one might expect malfunction to be a common event. However, the regulation is so exquisite, that, in fact, the system seldom fails. Nevertheless, failures do occur and when a stem cell escapes these fine control mechanisms, then unregulated cell proliferation may produce a vast overproduction of abberant cells - as in leukaemia.

1.4 LEUKAEMIA

1.4.1 Historical Perspective

It seems reasonable to assume that leukaemia has been around as long as mankind himself. However, the disease was not properly described until the nineteenth century. The first record of a case of leukaemia is generally attributed to Alfred Valpeau in 1827 (106, 107, 108). The patient was a 63 year old florist who became ill in 1825 with a pronounced swelling of the abdomen, accompanied by fever and weakness. He died soon after admission to the hospital and at autopsy was found to have a greatly enlarged spleen. The blood was "thick, like gruel". Unfortunately, no microscopic studies were carried out, so, strictly speaking, these macroscopic changes cannot be attributed to an increased white blood cell count. The cause of this "paleness" of the blood remained unknown for almost twenty years, until cases of leukaemia with clinical, post-mortem, and microscopic findings were independently and almost simultaneously published in Edinburgh and Berlin.

In the Edinburgh Medical and Surgical Journal of October 1st 1845, two case reports appeared. In the first, David Craigie described a patient who had been under his care in 1841 (106). Autopsy revealed "globules of purulent matter" in the blood and massive hepatosplenomegaly. The second case was reported by John Hughes Bennett (1812-1875) (106). He noted that parts of the blood clot were unusually yellow, opaque, and dull in appearance and that material resembling thick pus could be squeezed from the cut ends of many veins. Both the spleen and liver were greatly enlarged. Microscopic examination of the blood clot revealed the presence of colourless corpuscles.

Some six weeks after Bennett's publication, Rudolph Virchow (1821-1902) reported from Berlin a patient whose blood vessels contained a "yellowish-white, almost greenish mass" (108). Microscopically, it contained "besides a very few red blood corpuscles.....the same colourless or white cells which also occur in normal blood". The relationship between red and white cells was the reverse of normal, so much so that Virchow coined the term "white blood" (weisses Blut) to describe the condition.

Despite the similarity of their findings, Bennett and Virchow chose to interpret them differently. Bennett thought that the colourless corpuscles were pus cells rather than white blood cells, and concluded that the disease was a "suppuration of the blood". Virchow, on the other hand, could find no inflammation that might have led to the formation of pus and was reluctant to call the condition "pyemia" (i.e. blood containing pus). Instead, he contented himself with the descriptive term "white blood". Some months later, in August 1846, having reviewed the current literature, Virchow rejected the pyemic theory of leukaemia (still called white blood). Instead, he took the view that the "pus corpuscles" were identical to the colourless bodies normally occurring in the blood, and that in leukaemia there was merely an increase in the normal number of these cells (108). The following year, in 1847, he proposed a new name for the disorder, namely "white blood" translated into Greek, i.e. "leukaemia" [λεκοσ (leucos = white), αιμα (haema = blood)]. This name did not meet with universal approval. Indeed, Bennett objected to the name "leukaemia" as a misnomer since the blood was not itself white. Instead, he proposed "leucocythaemia" (white-cell-blood) as a more satisfactory descriptive term. It is ironic, perhaps, to note that in retrospect "leucocythaemia" is probably the more accurate name, particularly since these colourless corpuscles came to be known as "leucocytes".

1.4.2 The Nature of Leukaemia

In simple terms, leukaemia is a cancer of the haematopoietic system. For one reason or another, a cell (or cells) escapes the complex controls that regulate normal

haematopoiesis. Some leukaemogenic event(s) occurs which endows a cell with a particular growth advantage which allows it to dominate over normal haematopoiesis.

Leukaemic cells do not develop fully to functionally normal end cells. Rather, they appear to undergo a "maturation arrest" at some point in their differentiation, and consequently accumulate as immature cells.

However, these immature forms do not necessarily display a phenotype that is identical to some normal counterpart (109). In a significant number of cases, a rather bizarre phenotype can be demonstrated whereby individual leukaemic cells co-express markers which are normally restricted to a single lineage - so called "lineage infidelity" (110). For example, there have been several reports of patients with acute myeloid leukaemia (AML) whose cells expressed nuclear terminal deoxynucleotidyl transferase (TdT) - TdT is normally expressed on early lymphocytic cells (111, 112, 113). In the majority of cases, however, it is possible to identify the predominant lineage and cell type involved, and so designate the leukaemia to a particular category, e.g. myeloid or lymphoid, acute or chronic.

1.4.3 The Aetiology of Leukaemia

It would be an over-simplification to speak of "the cause of leukaemia". Certainly, several diverse factors have been implicated in its aetiology and it is likely that some interaction between these factors will prove to be the effective cause.

(a) Heredity

It would appear that genetic factors may influence susceptibility to leukaemogenesis. This has been implied from experimental leukaemia in mice (114). Strains of mice have been developed by intensive inbreeding to produce animals that are genetically almost pure, i.e. identical. In some strains of mice, leukaemia can occur spontaneously in almost every individual. In other strains, leukaemia is rare. Clearly, some strains of mice have a genetic predisposition to developing leukaemia.

Corroborating evidence has come from human studies. One study estimated that among first-degree relatives of patients with either acute leukaemia or chronic lymphatic leukaemia, the incidence of leukaemia was 2.8 - 3 times higher than expected (115). The evidence suggested that the excess incidence was primarily due to a genetic predisposition in these families. In addition, the incidence of acute leukaemia in the identical twin of an afflicted patient is considerably higher than in the general population, and is extremely high if one twin develops the disease before the age of three (116). Furthermore, in children with some congenital defects, notably Down's syndrome, the incidence of leukaemia can be 20-fold higher than in unaffected children (117). Some rare hereditary diseases which are associated with chromosome breakage (e.g. Bloom's syndrome, Fanconi's anaemia, or ataxia-telangiectasia) carry a high risk of leukaemia (114,118).

It would seem, therefore, that although leukaemia is not inherited in a Mendelian fashion, nevertheless there can be some genetic predisposition. The overall contribution of this must, for the present, be regarded as small.

(b) Radiation

The most powerful demonstration of the leukaemogenic potential of ionizing radiation can be seen in the aftermath of the atomic bombs dropped on Hiroshima and Nagasaki in Japan in 1945. The high incidence of leukaemia in survivors of the explosions was ample evidence for a causative role for radiation in leukaemogenesis (119). In addition, there appears to be some evidence that patients receiving radiotherapy (e.g. for cervical cancer) may run a risk, albeit very small, of developing leukaemia (120).

(c) Viruses

Viruses were first implicated as a cause of leukaemia by Vilhelm Ellermann and Oluf Bang in Copenhagen in 1908 when they reported the cell-free transmission of chicken leukaemia (114). Although these studies were confirmed and extended, it

was over 40 years before similar successful experiments were performed on mammals. In 1951, in New York, Ludwik Gross showed for the first time that leukaemia could be transmitted to newborn mice by means of cell-free extracts, hence the Gross AKR leukaemia virus (121). Subsequently, viruses have been identified in a number of species; e.g. cats (feline leukaemia virus, FeLV (122)), cattle (bovine leukaemia virus, BLV (123)), and primates (gibbon ape leukaemia virus, GaLV (124)).

Although viruses can frequently be the causative agent in animal leukaemias, to date, in humans only two haematological malignancies have a viral association. Adult T-cell leukaemia (ATL) is associated with a C-type retrovirus, human T-cell lymphotropic virus type 1 (HTLV-1) (125) and Burkitt's lymphoma is associated with a large DNA virus, Epstein Barr virus (EBV) (126).

The precise way in which viruses may cause leukaemia in animals is still unclear, but it is likely that they will act in concert with other factors like radiation or chemicals. In common with most other cancers, leukaemia is multifactorial in its aetiology, and a succession of events will be necessary for the evolution of the malignant clone.

(d) Chemicals

There is a great belief that in our modern industrialized society, our polluted environment harbours many toxic substances. However, in humans, only benzene has been shown beyond reasonable doubt to be able to induce leukaemia (127). Its leukaemogenic potential was observed in an occupational setting (127). There is also some evidence that patients receiving cytotoxic drugs, like melphalan, for the treatment of cancer may show an elevated incidence of acute leukaemia (128).

1.4.4 Chemical Carcinogenesis

The first report of a possible connection between environmental chemicals and the induction of cancer appeared over two hundred years ago. In 1761, John Hill

(1716-1775) observed that nasal cancer occurred in people who used snuff excessively (129). Some years later, in 1775, Percival Pott (1713-1788) reported a high incidence of scrotal skin cancer in men who had spent their childhood as chimney sweeps and he correctly attributed this to their constant contact with coal tar and soot (130). A hundred years later, Bell observed skin cancer in the workers of the Scottish shale oil fields whose skin was in continuous contact with tar and paraffin oils (which we now know contain polycyclic aromatic hydrocarbons (PAH)) (131). By necessity, the nature of this relationship between chemicals and cancer remained obscure until the early part of this century.

One of the first successful attempts to chemically induce cancer in animals was made by Yamagiwa and Ichikawa in 1915 (132). Benign tumours (skin papillomas) and highly malignant carcinomas were evoked by painting the ears of rabbits with coal tar every 2-3 days for many months. In 1932, at the Research Institute of the Royal Cancer Hospital (Free) in London, the group led by Ernest Laurence Kennaway (1881-1958) demonstrated that a pure chemical compound of known molecular structure could cause cancer (133). The first pure hydrocarbon to be identified as a carcinogen was dibenz(a,h)anthracene. Subsequently, over the next six years, several other potent carcinogens were isolated, e.g. benzo(a)pyrene in 1933 (134), 3-methylcholanthrene in 1935 (135). In 1938, Bachmann and Chemerda succeeded in synthesizing 7,12-dimethylbenz(a)anthracene (DMBA) (136) and later that year Bachmann and Kennaway reported DMBA to be highly carcinogenic when skin-tested on mice (137). Even now, DMBA is still one of the most potent carcinogens known.

1.4.5 Experimental leukaemia

Although leukaemia is not one of the most common neoplastic diseases, it has nevertheless commanded an enormous amount of research into its causes and treatment. Indeed, the incidence of leukaemia is only of the order of 10 cases per

100,000 population (cf. breast 70 per 100,000; lung 45 per 100,000) (138). This figure represents only ~3% of all cancers (139).

Leukaemia has attracted much scientific attention because it represents a neoplastic disease of a rather unique organ - blood. The ease of access to the blood has made it a most convenient system to study. Individual cells are easily available. Normal / leukaemic cells can be readily identified, enumerated and manipulated. Information accrued from the study of leukaemia may have general application to other neoplastic disorders.

Obviously, leukaemia in humans does not lend itself to experimentation. There is an obvious attraction in any system which can reproducibly induce leukaemia in high yield in experimental animals. Such a system would offer a unique opportunity to study the leukaemogenic process.

Chemicals have been used for many years to induce experimental leukaemia in animals. One of the earliest reports was the induction of leukaemia in rats of the Wistar strain by tube-feeding them with the polycyclic aromatic hydrocarbon, 3-methylcholanthrene (140).

The work that will be presented here is concerned with particular aspects of a model developed almost thirty years ago, namely, the induction of leukaemia in rats of the Long Evans strain by multiple intravenous injections of the polycyclic aromatic hydrocarbon - 7,12 dimethyl-benz(a)anthracene (141).

In 1966, Huggins and Sugiyama (132) demonstrated that DMBA could elicit leukaemia in high yield in rats of the Long Evans strain given 4 intravenous injections at bi-weekly intervals. Several kinds of leukaemia were produced, but the most common type (~80%) was a diffuse hepatic form, often associated with erythroblastosis. Diagnosis was normally made by liver biopsy where the first sign was an infiltration of the sinusoids by densely staining basophilic cells. As the disease progressed, the infiltration became more pronounced until massive infiltration brought about liver failure and, subsequently, death. At autopsy, characteristically, the liver was greatly enlarged and had an unusual, mottled appearance. Often, the spleen, as

well, was enlarged. The leukaemia was shown to be transplantable. When whole blood from a leukaemic animal was injected intraperitoneally into Long Evans babes (24-48hr old), leukaemia developed in about 85% of the recipients.

It is possible to establish permanent cell lines from these leukaemic animals using either blood or spleen. Leukaemic blood, when injected subcutaneously into newborn rats of the same strain, may produce tumours which histologically resemble erythrosarcomas (142). By removing pieces of tumour, disrupting the tissue and suspending the cells in culture medium, permanent cell lines can be obtained (143).

1.4.6 *in vitro* studies using permanent cell lines

Cell studies *in vitro* have made a major contribution to our understanding of the cellular events in normal and leukaemic haematopoiesis. As well as allowing the characterization and enumeration of the various haematopoietic progenitors, cell culture techniques have also helped to delineate the regulatory factors controlling both normal and leukaemic haematopoiesis.

Leukaemic cells appear to undergo maturation arrest, i.e. normal differentiation is blocked (109). A variety of leukaemic cell lines have been established both from humans and rodents, and these have provided a means for directly analysing the factors that influence proliferation and differentiation of these cells. This list of cell lines is extensive, but it would be inappropriate to review these data here. Among the most extensively studied human cell lines are HL-60, which was derived from a patient with acute promyelocytic leukaemia (144) and K-562 which was established from the pleural effusion of a patient with CML in blast crisis (145). Rodent cell lines include MELC, which are murine erythroleukaemic cells transformed by Friend virus (146), and WEHI-3B which were derived from murine myelomonocytic leukaemia cells (147). These cell lines have been used in many studies of the differentiation process. Indeed, MEL cells have several similarities with the REL cells under study in this work. It was a serendipitous discovery by Charlotte Friend in 1971 that showed that these MEL cells could be induced to differentiate

along an erythroid pathway following exposure to dimethylsulphoxide DMSO (148). Subsequently, Rifkind and Marks' group showed that this inductive effect could be reproduced by a variety of chemicals, including N-methylformamide and N-methylacetamide (149), the common feature of the molecules being that they were polar-planer in their configuration. In a further study, they reported a new group of potent inducers, the most effective being hexamethylenebisacetamide (HMBA) (150). Using this model, an impressive body of knowledge has accumulated about the process by which proliferating precursor cells can be induced to withdraw from the cell division cycle and, instead, express those characteristics of the differentiated phenotype.

Similarly, HL-60 cells can be induced to differentiate by a variety of agents such as DMSO (151) and retinoic acid (152), however the induced phenotype is granulocytic rather than erythroid. Furthermore, on exposure to agents such as phorbol myristate acetate (TPA) (153) or cytosine arabinoside (ara-C) (154), HL-60 cells can differentiate into macrophage- or monocyte-like cells, demonstrating a bipotentiality. In addition, there is some evidence that HL-60 cells, maintained in slightly alkaline medium, can develop granules characteristic of eosinophils (155), suggesting that they may even be tripotential. HL-60 cells, therefore, provide a model for investigating the mechanisms that regulate differentiation, especially the "decision -making" process that determines which lineage will be persued.

The validity of using these established cell lines to elucidate the nature of the leukaemic lesion is indicated by the fact that they can be induced to differentiate into cells with many of the characteristics of normal mature leukocytes (156). Furthermore, this suggests that these leukaemic cells have retained much of the cellular apparatus required to express the mature end-cell phenotype. Therefore, the aberrations of this maturational programme found in these cell lines are likely to be similar to the dysregulation that occurs *in vivo*.

From a clinical point of view, the goal of all this work is to exploit the experimental findings so that patient care may be improved. This indeed has proved to

be the case. Differentiation therapy has been applied effectively to achieve complete remission (CR) in cases of acute promyelocytic leukaemia (APL) using all-trans-retinoic acid as the inducing agent (157). Similarly, HMBA has been used to achieve partial or complete remissions, in terms of measurable tumour regression, in 5 of 33 patients with solid tumours (158), and in 6 of 20 patients with myelodsplastic syndrome (MDS) or acute myeloid leukaemia (AML) (159). These sorts of data are the ultimate demonstration that these experimental systems can make a valid and worthwhile contribution to our understanding of the regulation of haematopoiesis.

SECTION 2

MATERIALS AND METHODS

2.1 Induction of Leukaemia

Leukaemia was elicited in randomly-bred, weanling (4-6 weeks) rats of the Long Evans strain by serial administration of the polycyclic aromatic hydrocarbon, 7, 12-dimethylbenz (a) anthracene (DMBA) (Upjohn, Kalamazoo, Michigan, USA) (141). Rats received 4 intravenous injections of a lipid emulsion of DMBA (35mg/kg) at bi-weekly intervals. Prior experience showed that about 40% of the rats developed leukaemia, often within a short period of time (<100 days). The diagnosis of leukaemia was made by histological examination of liver, obtained by open biopsy under brief ether anaesthesia, commencing two weeks after the last dose of carcinogen and repeated at 14 day intervals. The earliest evidence for the induction of leukaemia was the appearance of aggregates of basophilic cells in the liver sinusoids. Over subsequent days and weeks, this infiltration became extensive and frequently involved the spleen.

2.2 Preparation of Culture Medium

The culture medium used throughout this study was Ostertag's modification of Eagle's Medium i.e. Modified Eagle's Medium-Ostertag i.e. MEM-O.

The overall formula for MEM-O was:-

MEM Amino acids (50x) (MEM AA)	: 40ml/litre
MEM Non Essential Amino Acids (100x) (MEM NEAA)	: 20ml
MEM Vitamins (100x)	: 20ml
MEM Sodium Pyruvate (100 mM)	: 10 ml
Penicillin (5000U/ml)-Streptomycin Solution (500µg/ml)	: 20 ml
Sodium Bicarbonate (7.5%)	: 25 ml
L-glutamine (200 mM)	: 20 ml
Foetal bovine serum (FBS)	:100 ml

All the reagents were obtained from Life Technologies, Paisley, UK.

The volume was made up to 1 litre with Earle's Balanced Salts Solution (EBSS) (i.e. 745ml).

Because of their instability in solution at temperatures above -10°C, L-glutamine and FBS were normally omitted when preparing batches of medium. L-glutamine and FBS were stored at -20°C and thawed out and added to the culture medium as required.

In practice, it was more convenient to prepare the medium by adding, aseptically, the appropriate volumes of ingredients to a commercially produced 500 ml bottle of EBSS.

To a 500 ml bottle of EBSS was added: -

MEM AA	: 26.8 ml
MEM NEAA	: 13.4 ml
Vitamins	: 13.4 ml
Pyruvate	: 6.7 ml
Pen/Strep	: 13.4 ml
Na HCO_3	: 16.8 ml

The pH was adjusted to pH 7.2-7.4 by dropwise addition of 4N NaOH. Since this preparation lacked L-glutamine and FBS it was referred to as MEM-O=. Complete medium, i.e. MEM-O++, was prepared by adding to 135ml of MEM-O= in a sterile medium bottle:-

L-glutamine	:	3 ml
FBS	:	15 ml

This gave a final volume of 150 ml of complete medium, MEM-O++. The final FBS concentration was 10% (v/v) (see 3.1). In addition, MEM-O± was occasionally prepared. This consisted of MEM-O= with added L-glutamine, but no added FBS, i.e. MEM-O±. This was used, for example, in the preparation of methyl cellulose for the in vitro clonogenic assay (see Section 2.5.1).

2.3 Establishment of Cell Lines

Rats diagnosed as being leukaemic were sacrificed by cervical dislocation and, under sterile conditions, the spleen was removed to a 90 mm Petri dish (Sterilin). Using a 23G needle (1¼, microlance Becton Dickinson) and syringe (Plasti-Pak, Becton Dickinson), the spleen was perfused with 20 ml of MEM-O++. Aliquots of the resulting cell suspension were seeded into a number of 25 cm^2 tissue culture flasks (Nunclon, Life Technologies, Paisley, UK) at various cell concentrations:- 1 x 10^3/ml; 1 x 10^4/ml; 1 x 10^5/ml. The culture medium was MEM-O++.

The cultures were routinely maintained at a cell density between 0.05 x 10^6/ml and 2 x 10^6/ml at 37°C/5% CO_2 in a fully humidified atmosphere. These wild cells were used to clone cell lines.

2.3.1 Cloning of cell lines

Wild cells were prepared in MEM-O++ at a nominal concentration of 5 cells/ml. Aliquots of 100µl were added to each well of 96-well, low - evaporation tissue culture plates (flat bottom) (Costar). This represented aliquots of, nominally, "0.5 cell/well". The plates were incubated for 4 days at 37°C/5% CO_2 in a fully humidified atmosphere.

Individual wells were examined using an inverted microscope (Labovert, Leica). Positive wells (i.e. containing cells) were easily identified and the contents of individual wells were each transferred by Pasteur pipette to a 25 cm^2 tissue culture flask containing MEM-O++. These cultures were maintained under the usual conditions until the cell concentration was of the order of 1-2 x 10^6/ml. At this point the cloning procedure was repeated. Only then were cells from a positive well deemed to be a clone. Clone REL-C7 was used throughout this work (**R**at, erythro**l**eukaemia - **c**lone number 7). This particular clone was chosen for its ability to undergo erythroid differentiation on incubation with dimethylsulphoxide (DMSO).

2.4 Cryopreservation of clones

The many cell lines produced by the cloning procedure were conveniently cryopreserved and may be stored for long periods of time (>5 years) in liquid nitrogen.

Cells from exponential-growing cultures were gently centrifuged (100g/10min) (Centra 3, Damon/IEC). The resulting cell pellet was resuspended in 1 ml MEM-O++ and then additional medium was added to adjust the cell concentration to 10 x 10^6/ml. The cells were placed on ice.

Dimethylsulphoxide (DMSO, Sigma) was used as cryoprotectant. DMSO was prepared at 20% (v/v) in MEM-O++ on ice. Equal volumes of cell suspension and DMSO solution were mixed and 2ml aliquots dispensed into Cryotubes (Nunc, Life Technologies, Paisley, UK) on ice. The final DMSO concentration was now 10% and the final cell concentration was 5 x 10^6/ml. The cryotubes were transferred to a polystyrene container and placed in a -80°C freezer overnight. The aliquots were subsequently transferred to the vapour phase of a liquid nitrogen storage system (K Series, Cryostorage System, Jencons). In this particular case, the polystyrene container consisted of two polystyrene trays in which 15 ml centrifuge tubes (Bibby, UK) had been supplied. The cryotubes were placed in the slots of one tray and the second tray was placed on top to form an enclosed box. The two trays were secured together with labelling tape (Whatman, UK). This simple procedure avoided the need for a controlled rate freezer for cryopreservation (160).

2.4.1 Recovery of cryopreserved cell lines

There is not "one right way" to recover cells from cryopreservation. Depending on the cell type and method of freezing, different laboratories have developed techniques that are appropriate for their own use.

Aliquots were recovered from the vapour phase of liquid nitrogen and rapidly thawed by placing in a 37°C water bath. Immediately, the cryotube was removed from the water bath and the contents transferred to a 15 ml centrifuge tube (Bibby,

UK) containing 1ml cold MEM-O++. The subsequent steps in cell recovery were performed on ice. A total of 10 ml of cold medium was added in a stepwise fashion of 10 x 1ml aliquots given at intervals of 2-3 minutes. This addition of medium over 20-30 minutes allows the intracellular DMSO to leave the cell slowly, thus reducing the level of DMSO-induced membrane damage.

When the rehydration process was complete the cells were centrifuged (100g / 10min). The resulting cell pellet was resuspended and washed in MEM-O++ (100g / 10m). After resuspension, these cells were ready for use.

Cell viability was determined by Trypan Blue exclusion (Life Technologies). Cells and Trypan Blue were mixed in the proportions 4:1, e.g. 4 drops of cell suspension and 1 drop of Trypan Blue were deposited in a bijou bottle, and left to stand at room temperature for 5 min. A small volume of culture/dye was removed and introduced into a haemocytometer counting chamber. Simultaneous counts were made for non-viable cells (staining blue, since the dye had penetrated the cell membrane) and for viable cells (colourless, since the intact cell membrane prevented penetration of the dye). A total of 400 (approx) cells was counted and the percent viable was calculated:-

$$\frac{\text{No. of viable cells counted} \times 100\%}{(\text{No.of viable cells}) + (\text{No.of non-viable cells})}$$

Generally, there was some variation in the percentages of viable cells recovered from cryopreservation. This ranged from 10%-40% viability, perhaps reflecting the fragile nature of cultured cells.

2.5 Invitro clonogenic assay

A semi solid culture system was developed for clonogenic studies. This involved suspending cellsin MEM-O= containing 0.9% methylcellulose (Methocel, Fluka) and 20% FBS.

2.5.1 Preparation of methyl cellulose

Methyl cellulose (High Viscosity, USP Methocel, 3000-5000 mPa.s, Fluka) was prepared in batches of approximately 500 ml by adding 10.725g to a 1 litre

Erlenmeyer flask containing a large (e.g. 60mm) magnetic stirring bar. The flask was sealed and autoclaved (15 psi, 20m). As a sterilisation check, an indicator tube was always included with the flask (Sterilizer Control Tube Type 1, Albert Browne Ltd., Leicester). Two hundred and fifty millilitres of sterile double distilled water was pre-heated to 90°C and added to the flask, stirring continuously. To avoid formation of clumps and ensure proper dispersion of the methyl cellulose, the suspension was stirred constantly.

Stirring was continued until the flask had cooled to hand-hot (~50°C) (1.5-2h). An equal volume (~250ml) of double strength MEM-O+ was added. The flask was then transferred to the cold room and kept overnight at 4°C, stirring continuously. After stirring overnight, the medium appeared perfectly clear. If it was at all turbid, it was discarded. The medium was now viscous (e.g. the stirring bar was no longer able to turn). From the flask, aliquots of 20 ml were poured into sterile Universal containers (Sterilin). As a precaution, these Universals were stored in the dark at room temperature for 1 week. If there were no signs of contamination, the methyl cellulose was stored at -20°C until required (< 1 year). When required for use, the methyl cellulose was thawed in a 37°C water bath. Once thawed, the methyl cellulose was used within one week, then discarded.

2.5.2 Clonogenic Assay

Depending on the number of tests to be set up, appropriate volumes of methyl cellulose and FBS were mixed in the proportion 2:1. Using a 2 ml syringe, aliquots of 1.8 ml were dispensed into 12 x 75 mm tubes (Falcon 2058) and the volume made up to 2.4 ml with MEM-O++ containing cells. The tubes were capped, shaken vigorously and the contents allowed to settle for 2 min (no longer than 5 min). Using a 1 ml syringe, aliquots of 1 ml were dispensed into each of 2 suspension cultures dishes (35mm) (Lux Suspension Culture Dishes, Flow Laboratories). These were then placed inside a larger Petri dish (90mm) (Sterilin) together with a third small dish (30mm) (Sterilin) containing 4 ml sterile distilled water to reduce

evaporation. The plates were incubated at 37°C / 5%CO_2 / humidified atmosphere for 4 days. Colonies were enumerated under an inverted microscope (Diavert, Leica). Usually, the entire plate was examined, using a grid to facilitate counting.

Example : For 4 tests:-

methyl cellulose	: 6 ml
FBS	: 3 ml
Total	: 9 ml →→→ 4 x 1.8 ml aliquots in Falcon tubes.

Each tube still requires a further 600 µl to make the final volume 2.4 ml.

REL-C7 cells at 0.2 x 10^6/ml. To each tube, add 12 µl (≡ 2.4 x 10^3) of cells. Each tube now requires to be made up to 2.4 ml. For example, the control tube would receive 588 µl of MEM-O++ to make up the final volume to 2.4 ml. Alternatively, if "substance x" was being tested (at, say, 10% concentration) then that tube would receive 240 µl of substance x + 348 µl of MEM-O++. The final cell concentration, in this example, is 1 x 10^3/ml. Routinely, this cell concentration was found to be the most convenient for subsequent colony counting. Clearly, the ingredients of each tube can be readily adjusted to investigate different cell concentrations, different factors, etc...

2.6 In vivo Tumourogenic assay

The malignant nature of the cell lines was investigated in an in vivo tumourogenic assay. The recipient rats were Long Evans babes between 3 and 7 days old. Each animal received a sub-cutaneous injection (Becton Dickinson, Microlance,"brown needle", 26G,3/8) of cells in a volume of 0.1 ml, administered under the loose skin behind the head in the scruff of the neck. A positive result was deemed to be the development of a tumour (tumour size, ~10mm) at the site of injection. As soon as tumour formation was confirmed, the animals were destroyed by exposure to ether in a closed container until they succumbed. For numbers of cells required, time taken for tumour formation, etc, see Results, Section 3.3.

2.7 Chemically-induced differentiation

For the differentiation studies, tissue culture flasks containing medium were pre-equilibrated by incubating them at 37°C/5%CO_2/humidified atmosphere for 2-3 hours before use. Several compounds were tested for their ability to induce differentiation in REL-C7 cells.

2.7.1 Dimethyl sulphoxide (DMSO)(Sigma)

Routinely, DMSO was used at a concentration of 1.5% (v/v) (equivalent to 211 mM) (see Results Section). The appropriate volume of DMSO was always added to the culture medium prior to the addition of cells. This precaution was taken to prevent any damage to the cells from the rise in temperature due to the Heat of Solution, released when an aqueous phase (medium) and a non-aqueous phase (DMSO) are mixed. For example, to 5 ml of equilibrated medium, 75 µl of DMSO were added (i.e. 1.5%). Subsequently, cells were added (say 5 x 10^4cells into 5 ml medium/DMSO to give 1 x 10^4/ml).

2.7.2 Hexamethylene bisacetamide (HMBA) (Aldrich)

A stock solution of HMBA was prepared at 50mM prior to each experiment (HMBA M.Wt.200). Test concentrations investigated were in the range 1-5mM. HMBA was dissolved in MEM-O++ and filter sterilised (Millex-OR, 0.22 µm pore size, Millipore). Since HMBA has a tendency to become hydrated in aqueous solution and consequently lose its activity, it was prepared fresh as required and used immediately.

2.7.3 all trans-Retinoic Acid (ATRA) (Sigma)

A stock solution of ATRA (M.Wt. 300.4) was prepared in ethanol (Analar, Burroughs) at 10^{-2} M. ATRA was examined for its ability to induce differentiation over the range 10^{-5}M - 10^{-8}M. To avoid any possible toxic effects of ethanol on the

cell cultures, the volume of ATRA solution added was kept to a minimum, e.g. 5µl ATRA into 5 ml cell cultures (1:1000).

2.7.4 Sodium butyrate (NaBu) (BDH)

A stock solution of NaBu was prepared at 20mM in MEM-O++ (M.Wt. 110.09), and filter sterilised before use. The concentration range investigated was 0.05mM-5mM.

2.7.5 N-Methyl Formamide (NMF) (BDH)

NMF (M.Wt. 59.07) is supplied at 17.12M. Appropriate dilutions were made in MEM-O++ to investigate the range 10^{-4}M - 10^{-1}M.

2.7.6 Cytosine Arabinoside (ara-C) (Sigma)

Ara-C (M.Wt. 243.21) is supplied in a vial of 100 mg. This was dissolved in 4.12 ml of the diluent provided to give a stock solution of 10^{-1}M. Subsequent dilutions were made in MEM-O++. Ara-C was tested over the range 10^{-9}M - 10^{-4}M.

2.8 Assessment of differentiation

Differentiation could be assessed by:-

1. Morphology.
2. Demonstration of the appearance of haemoglobin by the benzidine reaction.

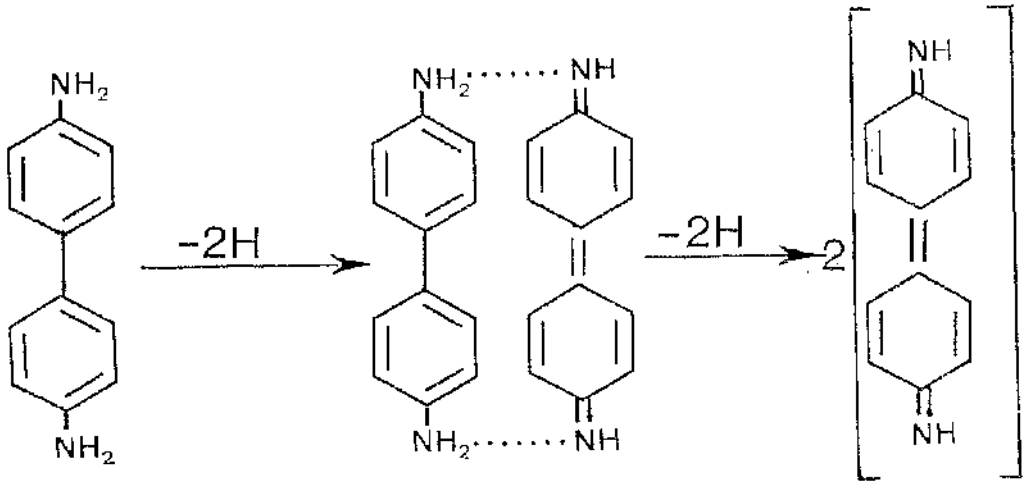
2.8.1 Morphology

After appropriate exposure to the inducing agent (eg DMSO), a Cytospin preparation was made of the cells. Routinely, a small aliquot of cells was removed from culture and resuspended in HBSS to a concentration of 1 x 10^4/ml. A 0.5ml aliquot (= 5 x 10^3 cells total) was dispensed into the slide holder of a Cytospin (Cytospin 2, Shandon). The cell preparations were spun at 500 rpm for 5min. Cell lines in general, and these differentiated cells in particular, are rather fragile and slow

speeds must be employed with the Cytospin to minimise cell damage (see Section 2.4.1, recovery of cryopreserved cell lines). The slides were recovered, allowed to air-dry and were then stained with Wright's Stain in a staining machine (Hematek, Ames). The slides were mounted in DPX and the cells examined by light microscopy (Dialux, Leica) at low (100x) and high power (400 x).

2.8.2 Benzidine Reaction

The benzidine reaction is the oxidation of benzidine by the peroxide - peroxidase system to a blue or brown product (161):-



colourless **blue** **brown**

The intermediate compound, benzidine blue, is unstable and is oxidised to the brown colour without enzymatic intervention. However, benzidine blue can be stabilised by acidification of the benzidine-peroxide medium, resulting in a colour that is predominantly blue rather than brown. Since haemoglobin is a catalyst for the oxidation of benzidine, then the appearance of benzidine blue is a rapid and sensitive indicator of haemoglobin production.

The benzidine stain for the detection of haemoglobin was used to determine the percentage of erythroid differentiated cells. At daily intervals 1 ml of culture was removed and transferred to an Eppendorf tube (1.5ml, Sarstedt) and centrifuged at 100 g for 5min. The supernatant was carefully aspirated and the pellet gently resuspended in 0.2ml HBSS. To this was added an equal volume of the benzidine reagent.

The benzidine reagent consisted of benzidine hydrochloride and hydrogen peroxide (H_2O_2). Benzidine hydrochloride (Sigma) is supplied in an Isopac bottle containing 1 mg. To this was added 50ml of 0.5% acetic acid. Benzidine and H_2O_2(30% solution, Sigma) were mixed in the ratio 1 part H_2O_2 to 99 parts benzidine in a bijou bottle (Sterilin).

After the addition of benzidine reagent to the cells, the percentage of haemoglobinized cells was determined within 5 minutes. A small aliquot was taken up in a capillary tube (Scotlab) and both chambers of a haemocytometer were filled (Improved Neubauer, Hawksley). The cells were examined under low power (100x) light microscopy (Dialux, Lieca). Benzidine +ve cells were characterised by a strong blue colouration; benzidine -ve cells appeared yellow. Routinely, at each assessment, both chambers of the haemocytometer were counted, approximately 400 cells per chamber. The percentage of benzidine positive cells was calculated.

2.8.2.1 Safety aspects of benzidine

Benzidine has been officially classified as a carcinogen (The Carcinogenic Substances Regulations 1967, Statutory Instrument No. 879). Although the use of benzidine is not actually prohibited, its use is restricted in accordance with the Health and Safety at Work etc.Act (1974). For the purpose of this study, a certificate of exemption from this restriction was obtained from the Health and Safety Executive Inspector of Factories (Exemption Licence No CAR/1985/1). This required that certain standards be met in the handling and disposal of benzidine.

Briefly, benzidine was stored under lock and key and its use was restricted to necessary personnel only. Benzidine was handled in a fume cupboard and the

operator wore protective clothing, the minimum being impervious gloves. Ehrlich's reagent (p-dimethylamino-benzaldehyde in methanolic hydrochloric acid) was used for routine decontamination of equipment and work surfaces as required, including counting chambers and microscope. Ehrlich's reagent was prepared by adding 25 g of -dimethylaminobenzaldehyde and 125 ml of 10 N hydrochloric acid to 2.5 litres of methanol (1% solution of p-dimethyl aminobenzaldehyde, 0.5 N hydrochloric acid).

When Ehrlich's reagent was used, e.g. to decontaminate counting chambers, a bright red-brown precipitate was produced. All gloves, tissues, bijou bottles etc used with benzidine were collected in plastic containers (Sharpsafe, Frontier Medical Products) and a specialist company was contracted to dispose of the waste safely (Leigh Environmental Ltd., Norwood Industrial Estate, Rotherham Road, Killamarsh, Sheffield, S31 8DR).

2.9 Commitment to differentiate by exposure to DMSO

When REL-C7 cells were exposed to 1.5% DMSO, maximal numbers of benzidine +ve cells were obtained on day 4 (see Results Section 3.4.1). Experiments were set up to investigate whether REL-C7 cells required to be exposed continually to DMSO to differentiate, or whether a brief exposure to DMSO was sufficient to commit the cells to differentiate in the subsequent absence of inducer. In any given experiment, multiple flasks of culture were set up, one flask for each time point.

Routinely, DMSO was used as the chemical inducer. The optimal concentration of DMSO that produced the highest percentage of haemoglobinized cells was 1.5% (v/v). Concentrations above 1.5% resulted in excessive cell death (See Results Section 3.5.1). Cultures were established by adding cells to medium/DMSO at a final concentration of 5×10^4/ml (see Section 2.7.1, Chemically-induced differentiation, DMSO). At appropriate time intervals, cultures were terminated by transferring the contents of a flask to a sterile 15 ml centrifuge tube (Corning) and centrifuging for 5 min at 100 g. The supernatant was poured off and the cell pellet gently resuspended in 1 ml of MEM-O++ (i.e. no DMSO). The volume was made up

to 10 ml and the cells centrifuged as before. This washing procedure was repeated one more time and the cell pellet was resuspended in 1 ml of MEM-O++ and counted in a semi-automatic counter (model M530. Coulter Electronics). Depending on this cell count, a portion of the cells was aspirated and the cell concentration adjusted to, nominally, 5 cells/ml (about 20-30 ml were usually prepared). Aliquots of 100 µl were dispensed into flat-bottomed 96-well tissue culture plates (Cel-Cult), i.e. "0.5 cell/well". The plates were incubated at 37°C/5%CO_2/humidified atmosphere, and were assessed 5 days later. Wells positive for growth were identified by light microscopy (Diavert, Leica). From these wells, as much of the culture supernatant as possible was carefully aspirated. Usually, about 90µl of supernatant could be removed without disturbing the cells. To each well, 100µl of the benzidine reagent was carefully added and the number of benzidine positive cells counted within 5 minutes by inverted light microscopy.

2.10 ^{3}H-Thymidine Incorporation following DMSO exposure

Cells were exposed to DMSO for various periods of time, then DNA synthesis was assessed by ^{3}H-thymidine incorporation.

Initial experiments investigated DMSO exposure times of 24hr, 48hr, 72hr, and 96hr. The experiments were planned so that all the cultures were harvested at the same time. Thus, flasks of culture were set up on consecutive days. To measure the rate of proliferation, cultures were exposed to ^{3}H-thymidine for the last 18hr of culture, prior to harvesting. The plan of the experiments can best be laid out thus :-

Day 0 : Time 10.00. Flask A : 1 flask of 10ml of REL-C7 set up at 0.01×10^6/ml in MEM-O++ / DMSO 1.5%.

Day 1 : Time 10.00 : Flask B : As above.

Day 2 : Time 10.00 : Flask C : As above.

Day 3 : Time 10.00 : Flask D : As above.

Time 16.00 : The contents of each flask were each transferred to a centrifuge tube and the cells spun down (100g / 5min).

The culture supernatants were aspirated and retained for subsequent use. The cell pellets were resuspended and a small aliquot of each was removed to perform a cell count. Using the appropriate previously retained culture supernatant, the cell concentration for each sample was adjusted to 0.5 x 10^6/ml. Aliquots of 200µl were dispensed into the wells of a 96-well flat bottomed tissue culture plate (Cel-Cult). Using a Hamilton Syringe (Capacity, 100µl) and a dispensor adaptor (50 strokes), aliquots of 2µl of ^{3}H-thymidine (TRA 120, Amersham) were dispensed into each well. ^{3}H-thymidine was supplied at 74 MegaBequerel (MBq) in a volume of 2ml. A stock solution was prepared : 0.2ml of ^{3}H-thymidine was added to 4.8ml of MEM-O++ to give 5ml of 1.48 MBq / ml. Each well received 2µl, which was equivalent to 0.003MBq.

Day 4: Time 10.00 : All cultures were terminated by harvesting the cells onto glass fibre discs using a Titertek Cell Harvester 530 (Flow Laboratories).

Thus, the cells from Flask A were cultured for a total of 96hr, continually in the presence of DMSO (Day 0, 10.00 →→→ Day 4, 10.00). Similarly, the cells from Flask B were exposed to DMSO for 72hr ; the cells from Flask C were exposed for 48hr ; and the cells from Flask D were exposed to DMSO for 24hr. For the final 18hr of these cultures, ^{3}H-thymidine was also present.

The discs were allowed to air-dry and then each disc was deposited into a numbered glass vial. Five millilitres of Ecoscint A scintillation solution (National Diagnostics) were added to each vial. The vials were then tightly capped and counted for 5 min on a Packard 300C β-counter. Control wells were included

which contained culture medium only. These control wells gave a measure of the background counts.

Results were expressed as counts per minute (cpm). The background cpm were deducted from all the test samples. Additional controls were set up which exactly duplicated these culture conditions, except that no DMSO was used. These cultures gave a measure of the amount of ^{3}H-thymidine taken up by normally proliferating cells. The cells in flask A, for example, were cultured for a total of 96hr without any replenishment of the medium. Although the starting cell concentration was low (0.01 x 10^{6}/ml), it is conceivable that some essential nutrients may become depleted over a 96hr culture. This would likely have an effect on the proliferative status of the cells, i.e. their growth would be retarded. Thus, parallel cultures (containing no DMSO) were set up to ensure that the level of proliferation was not adversely affected by the culture conditions themselves.

A second series of experiments was set up, investigating shorter DMSO exposure times, e.g. 2hr, 4hr, 6hr, and 8hr. The method outlined previously was modified to accomodate these shorter exposure times. Again the experiments were set up so that all the cultures were harvested at the same time. Thus, flasks of culture were set up at staggered time intervals, every 2 hours. In these experiments the cultures received a 90 min pulse of ^{3}H-thymidine (cf. 18hr pulse in the previous experiments). The plan of the experiments was:-

Day -1 : 10 flasks of 9ml of MEM-O++ were incubated overnight to allow the medium to equilibrate to the culture conditions.

Day 0 : Time 9.00 : Flask A : 1 flask of REL-C7 set up at 0.05 x 10^{6}/ml in MEM-O++ / DMSO 1.5%.
: 1 flask of REL-C7 set up at 0.05 x 10^{6}/ml in MEM-O++ alone (i.e. control)

: Time 11.00 : Flask B : As above.

: Time 13.00 : Flask C : As above.

: Time 15.00 : Flask D : As above, then immediately the contents of each flask were each transferred to a centrifuge tube and the cells spun down (100g / 5min). As before, each cell pellet was resuspended in the appropriate culture supernatant and the cell concentration was adjusted to 0.05 x 10^6/ml. Aliquots of 200µl were dispensed into the wells of a 96-well flat-bottomed tissue culture plate (Cel-Cult).

: Time 15.30 : All wells received 2µl of ^{3}H-thymidine (as before).

: Time 17.00 : All cultures were terminated by harvesting the cells onto glass fibre discs as described earlier. Thus, the cells from Flask A were cultured for a total of 8hr, continually in the presence of DMSO (9.00→→17.00). Similarly, the cells from Flask B were exposed to DMSO for 6hr; Flask C for 4hr; and Flask D for 2hr. For the final 90min of the cultures, ^{3}H-thymidine was also present.

The samples were counted as described earlier.

2.11 Cell Cycle Analyses

The cell cycle status was assessed by measuring the uptake and incorporation into DNA of a thymidine analogue, bromodeoxyuridine (BrdU). Cells can be pulse labelled with BrdU and those cells that are synthesizing DNA (i.e. in S-phase of cell cycle) will incorporate BrdU into the DNA. An appropriate anti-BrdU antibody is used to identify those cells undergoing DNA synthesis at the time of the pulse. The total DNA content of cells was assessed by propidium iodide (PI) uptake.

Reagents:

1. Normal Saline
2. Phosphate buffered saline (PBS)
3. Bovine serum albumin, 10% in PBS.
4. Tween 20, 0.5% in PBS.

5. Bromodeoxyuridine, (BrdU), 1mM stock solution in PBS, stored at -20°C.

6. Anti-BrdU (Becton Dickinson, Cat. No.7580).

7. Fluoroscein isothiocyanate (FITC) conjugated $F(ab')_2$ rabbit anti-mouse IgG (RAM IgG)

8. Propidium iodide, 20 μg/ml in PBS.

9. Hydrochloric acid, 4N.

10. Sodium Hydroxide, 0.07N.

11. Ethanol, 70% at -20°C.

12. Sodium tetraborate ($Na_2 B_4 O_7$), 0.1 M, pH 8.5.

Cells were cultured in the presence of DMSO as detailed earlier, except that the cell concentration was 0.1 x 10^6/ml. When the cells had been exposed to DMSO for the appropriate time, they were transferred to a 15 ml centrifuge tube, washed twice with MEM-O++ as before, and then seeded into 25 cm^2 tissue culture flasks containing 10 ml of equilibrated MEM-O++ with BrdU at 10 μM.

After a 30 min incubation, the cells were transferred to a centrifuge tube, and washed twice with PBS (Gibco) (100g/5min). The final supernatant was poured off and the cells resuspended in the small residual amount of supernatant remaining in the centrifuge tube (approx 100 μl). The cell suspension was chilled on ice (for about 5 min) and made up to 2 ml with cold 70% ethanol with vigorous mixing. The cells were fixed at 4°C for 30min.

To denature the cellular DNA and produce single stranded molecules, 2 ml of 4N HCl was added and the cell suspension was further incubated for 30min at room temperature. At this stage, the fixed cells become very sticky and great care was taken to avoid cell aggregates forming. For example, addition of HCl to the cell pellet will aggregate all the cells. The cells were centrifuged (100g/5min) and the pellet resuspended in 1 ml of 0.1 M $Na_2B_4O_7$ to neutralize the acid. Aliquots of 1 x 10^6cells were dispensed into Falcon tubes (Falcon 2058) and centrifuged (100g/5min). The supernatants were carefully aspirated and the pellets resuspended in

50 µl of 0.5% Tween 20/PBS. To each aliquot was added 20µl of anti-BrdU (Becton Dickinson Cat No 7580). Following a 30min incubation at room temperature, the cells were spun down (100g for 5min), then resuspended in 50 µl of 0.5% Tween 20/PBS. To each cell suspension were added 100 µl of a 1:10 dilution of FITC conjugated-RAM IgG (Dako). The cells were incubated for 30min at room temperature, spun down (100g for 5min) and the resulting pellets resuspended in 1 ml of PBS containing 5 µg/ml of propidium iodide (PI) (Sigma).

For each sample, 10,000 cells were analysed on the FACScan Flow Cytometry Analyzer (Becton Dickinson) using 488 nm excitation light. For two colour fluorescence, band pass filters 530/30 (for FITC) and 620/35 (for PI) were used in combination with a dichroic mirror 570. Cells were analyzed for their positivity to the BrdU antibody (FL1) and for their PI uptake (FL2).

In addition, control cells were analyzed to allow baseline settings to be entered into the FACScan prior to analyzing the test samples. Routinely, these control cells were peripheral blood lymphocytes from normal donors (e.g. laboratory personnel). Since lymphocytes in the circulation are normally non-proliferative, BrdU uptake should be minimal.

2.12 Assessment of cell size

All the flow cytometry was kindly performed by Mr. Charles Pearson, Haematology, GRI. Flow cytometry, for example using a FACScan, can be used both for analysis and separation of cells in suspension (162). As well as measuring the fluorescence of individual cells as they flow, single file, in a stream past a laser illumination system, flow cytometry can also measure cell size. FACS has a detector for forward light scatter (FLS) - this essentially measures cell size.

FACS uses the amount of light scattered at a small forward angle (1^{o}-13^{o}) as an index of cell size. This measure is mainly due to diffraction and is not strictly proportional to cell size because it is influenced by such cell properties as asymmetry, reflectivity, refractive index, granularity, and nuclear size. Nevertheless, there is a

strong positive correlation between cell size as measured by the FACS scatter detector and cell size as measured by sedimentation at unit gravity (163) or with the Coulter Counter (164).

REL-C7 cells were exposed to DMSO for various time intervals as detailed before (24hr, 48hr, 72hr, and 96hr). Cultures were set up at a concentration of 0.05 x 10^6/ml in a 5ml volume of MEM-O-+-+/DMSO 1.5%. After the appropriate period of exposure to DMSO, the contents of the flasks were each transferred to a 15ml centrifuge tube and the cells spun down (100g / 5min). The pellets were resuspended and washed in 5ml of Sheath Fluid (Becton Dickinson) (100g / 5min). This washing procedure was repeated and the cell pellet was resuspended and made up to ~1ml with Sheath Fluid. The cells were analyzed on a FACScan (Becton Dickinson).

For each sample, 10,000 cells were acquired and the FLS signal analyzed. FLS was amplified on a linear scale. The histogram profiles generated from each sample were overlaid together on the one display to allow direct comparison of relative cell sizes. Thus, any shift in cell size could be clearly demonstrated.

2.13 Scanning Electron Microscopy (SEM)

SEM was kindly provided by Mr J Anderson of the Department of Pathology, Glasgow Royal Infirmary. Briefly, following exposure to DMSO for various periods of time, REL cell suspensions were passed over a Millipore filter (25 mm diameter, 0.45 μm pore size) and the cells were retained on the filter surface.

With the filters still in the filter holders, the cells were fixed in 4% glutaraldehyde for 15min, rinsed briefly in distilled water, then dehydrated through 3 x 10 minutes changes of 100% ethanol. The filters were removed from the holders, mounted on card and transferred to a critical point drier (CPD) (Bio-Rad) for the final drying procedure in liquid CO_2. When the filters were completely dry, they were removed from the CPD, cut into strips, mounted onto copper stubs and coated with gold in a sputter coater (Bio-Rad). SEM was performed on a Joel 1200EX transmission microscope with a scanning attachment using an accelerating voltage of

80 kV. Images were recorded on Ilford FP4 film and processed using conventional darkroom techniques.

2.14 Adaptation of REL-C7 cells to serum- free culture.

Routinely, cells were cultured in MEM-O++ (i.e. MEM-O supplemented with 10% FBS). However, cultures could be readily adapted from serum- containing medium to serum- free medium.

Experiments were performed with two commercially available preparations:-

(a) SF-1 (Northumbria Biologicals)

(b) High Protein Hybridoma Medium (HPHM) (Gibco/BRL).

However, most of the data were generated using an in-house preparation which consisted of MEM-O= supplemented with ITS-Premix (Insulin; Transferrin; Selenious Acid) (Collaborative Research) at 0.1 % and bovine serum albumin (BSA) (Sigma) at 1%.

ITS-Premix comes as a lyophilised powder, supplied in a vial sufficient for 5 litres of medium. Each vial contained 25mg of insulin, 25 mg of transferrin and 25 µg of selenious acid. The powder was reconstituted with 5 ml of appropriate sterile serum- free culture medium. This stock solution was at 1000 X strength. Aliquots of 200 µl were dispensed into Nunc cryotubes and stored at -20°C until required. As stated above, the final working concentration of ITS-Premix was 0.1% (i.e. 1/1000-fold dilution) i.e. insulin 5 µg/ml, transferrin 5 µg/ml and selenious acid 5 ng/ml as per manufacturer's recommendations.

Bovine serum albumin (BSA) was de-ionized using analytical grade mixed bed resin beads (type AG501-X8(D), Bio-Rad). Ten grammes of BSA were dissolved in 44 ml of double distilled water. Approximately 1 gm of resin beads was added, and the solution was gently mixed on a Roller Mixer (Model CB 5150, Denley) for 1 hour. The resin beads changed colour from blue to yellow. The exhausted beads were removed by centrifugation (400 g/10min). The de-ionization procedure was repeated by again adding 1 gm of resin beads and mixing gently for 1 hour. Usually at this

stage the de-ionization procedure was complete as demonstrated by the fact that while some beads were exhausted (yellow), others remained blue. As before, the resin beads were removed by centrifugation. To this de-ionized BSA was added an equal volume of double strength MEM-O± :-

MEM AA	: 4.0ml
MEM NEAA	: 2.0
Vitamins	: 2.0
Pyruvate	: 1.0
Pen/Strep	: 2.0
Na HCO_3	: 2.5
L-glu	: 2.0
EBSS (10x)	: 8.45
Distilled water	: 26.05
Total	**: 50.0 ml** of double strength MEM-O±.

The resulting solution was approximately 10% BSA (w/v). The solution was sterilised through a Millipore disposable filter ("Millex-OR", Pore size 0.22 μm). Aliquots of 5 ml were dispensed into sterile bijou bottles and stored at -20°C until required.

Cells were adapted to serum- free conditions by stepwise reduction of the serum concentration in the culture medium. For example, cells originally maintained in MEM-O++ were spun down (100 g for 5min) and washed twice with a mixture of MEM-O++ and, say, SF-1 in the ratio 1:9 (i.e. 1%FBS). After the second wash, the cells were resuspended in this medium mixture at an appropriate concentration (5 x 10^4/ml) and were maintained under the usual culture conditions for 48 h to allow the cells to adjust to this reduction in FBS. After this time interval, the process was repeated using a medium mixture of MEM-O++: SF-1 of 0.5:9.5 (i.e. 0.5% FBS). This procedure was repeated through several cycles to bring the FBS concentration down consecutively to 0.25%, 0.10% and finally 0.05% FBS (i.e. a 200 fold reduction

in the FBS concentration from the original culture conditions). At this stage, the cells were washed twice, as before, with serum-free medium (eg SF-1) and resuspended at 5 x 10^4/ml in that serum-free medium.

2.15 Determination of doubling-time for REL-C7 cells

After cells had been weaned off serum and adapted to serum free culture conditions, it was important to ensure that cells could still proliferate at an acceptable level. For this reason, growth curves were constructed for cells grown under various culture conditions.

From established cultures, growing exponentially, cells were aspirated (5-10ml) and spun down (100 g for 5min). The supernatant was poured off and the cell pellet gently resuspended in the residual supernatant (0.3 ml approx). A small portion of this was counted in a Coulter Counter. An appropriate volume of cells was added to duplicate flasks of equilibrated medium to give a final concentration of 0.05 x 10^6/ml. At various time intervals thereafter, a small portion of the culture was aspirated and introduced into a haemocytometer counting chamber. All four "corner squares" were counted and the cell concentration calculated. This procedure was repeated over five days to build up a graph of increasing cell numbers versus time. Doubling times were calculated for various culture conditions.

For example:

Time 0 : cultures established at 0.05 x 10^6/ml

Time 18 h : cell count 0.08 x 10^6/ml

Time 84 h : cell count 1.3 x 10^6/ml.

Cell doubling : 0.08 x 10^6/ml.
0.16
0.32
0.64
1.28 x 10^6/ml.
} 4 doublings of cell numbers in 62 hr (84-18)

i.e. 4 doublings in 62 hr ≡ doubling time of 15.5 hr. The calculation of these doubling times allowed direct comparisons to be made between various media for their ability to support cell proliferation.

2.16 Substitution of FBS by various sera

To further investigate the role of serum in the maintenance of REL-C7, cultures were adapted from the usual FBS containing medium to MEM-O containing alternative sera. These sera were:-

a) Mouse

b) Rat

c) Rabbit

d) Horse

e) Human

Blood from mice and rats was obtained by cardiac puncture of anaesthetised animals (Animal Unit, University Department of Pathology, GRI). Rabbit blood was obtained by ear-bleed. In all cases, the bloods were dispensed into serum bottles (L.I.P.Ltd., Shipley, West Yorkshire), allowed to clot and spun down (400g for 10min). Serum was carefully aspirated, filter sterilized (Millipore, "Millex-OR, pore size, 0.22μm) and stored at -20°C until required.

Horse serum was commercially available (Life Technologies). Human serum was available from an "in-house" pool of sera from normal donors, namely, laboratory personnel. Cells were adapted to these alternative sera in a similar fashion to the adaptation to serum- free conditions, i.e. progressively reducing the FBS concentration in the culture medium, while simultaneously increasing the concentration of the particular alternative serum. The total serum concentration was maintained at 10% at all times. Cells readily adapted to alternative sera without any loss of viability or extension to their doubling time (see Section 3.6.4).

2.17 Modification of FBS by heat treatment

In a further attempt to define more precisely the role of FBS in the proliferation of REL-C7 cells, FBS was modified by 2 methods of heat treatment.

2.17.1 Heat inactivation

Aliquots of 20 ml of FBS were dispensed into sterile Universal containers (Sterilin). These were then placed in a water bath and incubated at 56°C for 30min. This FBS was then deemed to be "heat inactivated".

2.17.2 Denaturation

Aliquots of 10 ml of FBS were dispensed into sterile glass Universal containers (McQuilkin). These were then plunged into boiling water in a Dental Steriliser (Surgical Equipment Supplies Ltd., London) for 5 min. Some pale floccules often precipitated out. This precipitate was allowed to settle out and the FBS supernatant, now pale yellow, was aspirated and used in subsequent investigations. This FBS supernatant was deemed to be "denatured FBS".

2.18 Addition of recombinant growth factors to serum-free medium

To try to identify some of the crucial activities that regulate commitment and differentiation, serum-free media were prepared (Section 2.14) which were supplemented with a range of cytokines / growth factors. The basic serum-free medium was the in-house preparation, MEM-O±/ITS, BSA. Prior to investigating the effects of added cytokines, the REL-C7 cells were adapted to this serum-free medium as outlined in Section 2.14.

The cytokines investigated were :-

(a) human Interleukin-6 (IL-6),

(b) human Interleukin-11 (IL-11),

(c) human granulocyte colony stimulating factor (G-CSF),

(d) human macrophage colony stimulating factor (M-CSF),

(e) murine granulocyte-macrophage colony stimulating factor (GM-CSF),

(f) murine kit ligand (KLS),

(g) human macrophage inflammatory protein-1alpha (MIP-1α).

Note that these cytokines were of either human or murine origin rather than rat. This reflected the difficulty in obtaining rat recombinant cytokines. Where possible, murine cytokines were used since they would be most likely to be active on rat cells. Where murine cytokines were unavailable, human cytokines were used.

Some cytokines are species specific. Others show some cross species activity. For example, mouse and human IL-6 have 42% amino acid homology. Although murine IL-6 has no activity on human cells, human IL-6 is functional on murine cells (165). Thus, human IL-6 was presumed to be appropriate for use on rat cells. Similarly, human IL-11 is active on murine cells (166).

For G-CSF, there is about 73% amino acid homology between the human and murine proteins, and both act across species (167)

For M-CSF, there is about 82% amino acid homolgy between human and murine proteins in the N-terminal region. With this degree of homolgy it is rather surprising that murine M-CSF is not active on human cells. However, human M-CSF is active on murine cells (168).

For GM-CSF, there is about 56% amino acid homology between human and mouse, and no cross species activity (169). Fortunately, a source of murine GM-CSF was available.

For KLS (Stem Cell Factor), there is 79% amino acid homology between human and mouse. Although human KLS has only very weak activity on mouse cells, murine KLS is active on human cells (170). In any event, a source of murine KLS was available.

For MIP-1α, there is about 73% amino acid homolgy between human and mouse. The human and mouse proteins show cross-species activity (171).

2.18.1 Interleukin-6 (IL-6)

E.coli derived recombinant human IL-6 was obtained from Genetics Institute, Cambridge, Mass.,USA at a concentration of 22μg/ml. A stock solution was prepared by adding 0.5ml of IL-6 (22μg/ml) to 10.5ml of PBS/0.1%BSA to give a final concentration of 1μg/ml. This was dispensed into aliquots of 1ml in Nunc cryotubes and stored at -20°C until required. From the manufacturer's data sheet, the optimal working concentration was in the region of 20ng/ml. Thus, three concentrations of IL-6 were prepared :- the recommended concentration; a preparation which was a 10-fold dilution of this; and a preparation which was 10 times more concentrated than the recommended, viz., 2ng/ml, 20ng/ml, and 200ng/ml. These were all prepared by adding the appropriate volume of IL-6 (1μg/ml) to MEM-O±/ITS, BSA to obtain a final volume of 20ml.For example,

400μl of IL-6 (1μg/ml)
19.6ml of MEM-O±/ITS, BSA } 20ml of IL-6 at 20ng/ml.

2.18.2 Interleukin-11 (IL-11)

E.coli derived recombinant human IL-11 was obtained from Genetics Institute, Cambridge, Mass., USA. As above, a stock solution of 1μg/ml was prepared in PBS/0.1% BSA, and aliquots of 1ml were stored at -20°C until required. The recommended concentration was 10ng/ml from the manufacturer's data sheet. As before, a concentration higher, and a concentration lower than the recommended dose were both prepared, all in a final volume of 20ml of MEM-O±/ITS, BSA. Thus, the concentrations were 1ng/ml, 10ng/ml, and 100ng/ml.

2.18.3 Granulocyte colony stimulating factor (G-CSF)

Recombinant human G-CSF was a generous gift from Dr. T.L.Holyoake, Beatson Institute for Cancer Research, Glasgow. It was supplied as a conditioned medium (CM) from a Chinese Hamster Ovary (CHO) cell line engineered to express

human G-CSF. Optimal biological activity was obtained by using the CM at a final concentration of 10µl/ml. As before, three concentrations were prepared :- 1µl CM / ml, 10µl CM/ml, and 50µl CM/ml. All dilutions were made into MEM-O±/ITS, BSA.

It was felt inappropriate to prepare the highest concentration at 100µl CM/ml of cell culture, and instead, 50µl CM/ml of culture was prepared. This was because the culture medium in which the CHO cells had been maintained was supplemented with FBS at a final concentration of 10% (v/v). Had 100µl of this CHO conditioned medium been added to 900µl of MEM-O±/ITS, BSA, then the final product would have contained FBS at 1% (v/v). Earlier studies (Section 3.6.3) indicated that medium supplemented with FBS at 1% could support a low level of DMSO-induced erythroid differentiation. It was possible to get round this problem by preparing the highest concentration of G-CSF at 50µl CM/ml, resulting in a final FBS concentration of 0.5%. From previous results, this value was acceptable.

2.18.4 Macrophage colony stimulating factor (M-CSF)

Recombinant human M-CSF was obtained from Genetics Institute, Cambridge, Mass., USA. From the data sheet, the recommended working concentration was 10ng/ml. As before, three concentrations were prepared, each in 20ml of MEM-O±/ITS, BSA :-1ng/ml, 10ng/ml, and 100ng/ml.

2.18.5 Granulocyte-macrophage colony stimulating factor (GM-CSF)

Recombinant murine GM-CSF was obtained from Genetics Institute, Cambridge, Mass., USA as a conditioned medium (CM) prepared from cultures of COS-1 cells engineered to express murine GM-CSF. The activity of GM-CSF in the CM supplied was 16,300U/ml. A stock solution of 1,000U/ml was prepared by taking 0.613ml of GM-CSF (16,300U/ml) and making it up to 10ml with MEM-O±/ITS, BSA. This stock solution was used to prepare a range of concentrations :- 0.5U/ml, 5U/ml, and 50U/ml.

2.18.6 Kit Ligand (KLS)

Recombinant murine KLS (also known as Stem Cell Factor / Mast Cell Growth Factor / Steel Factor) was a generous gift from Dr. T.L.Holyoake, Beatson Institute for Cancer Research (BICR), Glasgow. It was supplied as a medium conditioned by CHO cells expressing soluble kit ligand. Titration studies at Genetics Institute indicated that 20µl of this CM had the equivalent activity of 12ng of recombinant purified material. Other cell culture studies at BICR indicated that a working concentration of 20µl of KLS-CM/ml culture (equivalent to 12ng/ml) produced maximum activity. For the studies here, three concentrations were prepared :- 2µl KLS-CM/ml (≡ 1.2ng/ml), 20µl KLS-CM/ml (≡ 12ng/ml), and 50µl KLS-CM/ml (≡ 30ng/ml).The highest concentration was 50µl/ml and not 200µl/ml so as to avoid the problem of FBS carry-over explained above in Section 2.18.3.

2.18.7 Macrophage inflammatory protein-1 alpha (MIP-1α)

Recombinant human MIP-1α was a generous gift from Dr. T.L.Holyoake, BICR, Glasgow. It was supplied as a conditioned medium prepared from cultures of COS-1 cells engineered to express human MIP-1α. Titration studies indicated that the concentration of MIP-1α was approximately 10µg/ml. In ongoing culture studies at BICR, a concentration of 200ng/ml gave maximum biological activity. As before, three concentrations were prepared in MEM-O±/ITS, BSA :- 20ng/ml; 200ng/ml; and 500ng/ml. The highest concentration of MIP-1α was 500ng/ml rather than 2,000ng/ml to avoid the potential effects of FBS carry-over.

2.19 Staining of adherent REL-C7 cells

Methods have previously been described for adapting REL-C7 cells to culture conditions that were serum-free, or contained serum at a reduced level (Section 2.14).When the concentration of FBS was reduced from 10% to 0.5%, a chance observation was made that REL-C7 cells no longer proliferated in suspension culture, but, instead, formed an adherent monolayer (Section 3.7). Under light microscopy, the

gross morphology of these adherent cells appeared different from suspension culture cells. Specialized staining procedures were performed to try to characterize these adherent cells :-

(a) Periodic Acid-Schiff (PAS),

(b) Sudan Black B,

(c) alpha-Naphthyl Acetate Esterase (α-NAE).

The stains were kindly set up by Mr.C.Pearson, Haematology, GRI, according to the routine departmental protocols. A brief description is given below.

2.19.1 REL-C7 cells from suspension culture

These were cells that had been routinely maintained under standard conditions in MEM-O++ as outlined earlier.

2.19.2 REL-C7 cells from adherent monolayer cultures

As described previously (Section 2.14), by a progressive step-wise reduction in the concentration of FBS, REL-C7 cells could be adapted to grow in MEM-O± /0.5% FBS. Prior to staining, the cells were recovered from the plastic surface of the tissue culture flask by vigourous pipetting. With a sterile, glass Pasteur pipette (Long form; McQuilkin, Glasgow) and a rubber pipette bulb, an amount of culture supernatant was drawn up into the pipette. This was then forcibly expelled at the adherent cells, thus releasing them from the plastic. An appropriate volume of supernatant, now containing cells in suspension, was removed for staining purposes.

2.19.3 Normal rat bone marrow cells

As a control, normal rat bone marow cells were set up. A rat of the Long Evans strain was sacrificed by cervical dislocation, and a femur excised. Femoral marrow cells were obtained by coring the shaft of the femur with a needle (25G) and syringe, and flushing out the marrow with 5ml of HBSS into a bijou bottle. The cells were disaggregated into a single cell suspension by repeated pipetting.

2.19.4 Preparation of Cytospins

Prior to staining, Cytospin preparations were made of each lot of cells (Cytospin 2, Shandon). In all cases, the cell concentration was adjusted to 2 x 10^5/ml in HBSS. The Cytospin chambers were assembled and an aliquot of 0.5ml of cell suspension was added to each (i.e. equivalent to 1 x 10^5cells per slide). To minimize any structural damage as the cells were deposited on the slides, slow speeds were used :- 500rpm for 5 min. The slides were then removed from the chambers and the cell "spots" allowed to air-dry.

2.19.5 Periodic Acid-Schiff (PAS)

The reagents used were mainly from the Sigma Kit 395-B.

1. Slides were placed in a rack and fixed in cold methanol for 15min, then washed for 5min in running tap water.
2. A solution of 1% periodic acid was prepared, and the slides were immersed in this for 10min. Again, they were washed in running tap water.
3. The slides were immersed in Schiff's reagent for 30min, washed in running tap water for 5min, and then air-dried.
4. The slides were counterstained with Haematoxylin for 5min, then "blued" by immersion in Scots Tap Water for 30sec (Scots Tap Water : 3.5g of sodium bicarbonate, 20g magnesium sulphate, dissolved in 1 litre of distilled water).
5. After the slides were air-dried, they were mounted in Depex.

2.19.6 Sudan Black B

The reagents used were mainly from the Sigma Kit 380B

1. Fixative was prepared by adding 25ml of acetone to 75ml of glutaraldehyde solution.
2. Slides were immersed in fixative for 1min on ice, with gentle agitation, and then washed in distilled water.

3. Slides were immersed in Sudan Black B Staining Reagent for 5min with intermittent agitation.
4. Slides were rinsed three times in 70% ethanol, or additional times until no more dye was washed out. They were then thoroughly rinsed in distilled water.
5. The slides were counterstained in Haematoxylin Solution (Gill No.3, GHS-3) for 5min, and then rinsed in tap water.
6. After air-drying, the slide were mounted in Depex.

2.19.7 alpha-Naphthyl Acetate Esterase (α-NAE)

The reagents used were mainly from Sigma Kit 91-A. Fixative was made up fresh before use. One millilitre of citrate concentrate solution was diluted in 9ml de-ionised water. Four millilitres of this citrate was added to 6ml of acetone. After thorough mixing, 9ml of this citrate:acetone was added to 1ml of methanol.

1. Slides were immersed in the above fixative for 30sec at room temperature, washed in de-ionised water and allowed to air-dry.
2. TRIZMAL^{TM} pH 7.6 buffer concentrate was diluted 1:10 by adding 5ml of buffer to 45ml of de-ionised water. To this 50ml of diluted buffer was added 1 capsule of Fast Blue BB Salt with constant stirring.
3. Two millilitres of α-naphthyl acetate were added to the mixture prepared in step 2.
4. This solution was then filtered through Whatman No.4 filter papers into a Coplin jar.
5. Slides were then immersed in this staining solution and incubated for 30min at 37^{o}C.
6. The slides were washed three times in tap water and counterstained for 10min in Mayer's Haematoxylin Solution.
7. Finally, the slides were washed in de-ionised water, air-dried and mounted in glycerol/gelatin

2.20 Immunophenotypic analyses of REL-C7 cells

Further characterization of REL-C7 cells was obtained by immunophenotypic analyses. This was done by incubating cells with a range of specific anti-rat monoclonal antobodies:-

Monoclonal Antibody	Supplier	Catalogue Number	Specificity
OX-1	Serotec	MCA 43G	Leucocyte common antigen (LCA) (CD45)
OX-4	Sera-Lab	MAS 029p	Immune associated (Ia)
OX-41	Serotec	MCA 274	Macrophage and granulocytes
OX-44	Serotec	MCA 371	All myeloid cells and peripheral lymphocytes (CD53)
OX-45	Sera-Lab	MAS 260p	Surface glycoprotein present on haematopoietic cells and endothelial cells (CD48)
ED-1	Serotec	MCA 341	Monocytes and macrophage

The Sera-Lab antibodies (OX-4 and OX-45) were supplied as 5ml of culture supernatant, ready to use. The antibodies were dispensed into aliquots of 250µl and stored at -20°C until required.

The Serotec antibodies (OX-1, OX-41, OX-44, and ED-1) were supplied as 0.25ml of ascitic fluid. Stock solutions were prepared by adding this 0.25ml volume to 2.25ml of PBS / 0.5% BSA (i.e. a 1:10 dilution). The stock solutions were dispensed into aliquots of 200µl in Nunc cryotubes and stored at -20°C until required. Prior to use, an aliquot was thawed, and diluted 1:10 again, giving approximately 2ml of working concentration antibody.

As outlined in the previous sections (2.19.1, 2.19.2, and 2.19.3), three lots of cells were analyzed:-

(a) suspension culture REL-C7 cells,

(b) adherent monolayer REL-C7 cells,

(c) normal rat bone marrow cells.

For each cell type, cell suspensions were prepared at a concentration of 5 x 10^6/ml in PBS.

1. To each tube (Falcon 2058, A.& J. Beveridge) was added 200µl of cell suspension (i.e. equivalent to 1 x 10^6cells).
2. Fifty microlitres of the appropriate antibody was added, then the contents were thoroughly mixed and placed on ice for 30min with frequent (every 5min) agitation.
3. The tubes were topped up with cold PBS (~4ml) and spun for 10min at 100g in a refrigerated centrifuge at 4°C (Centra 7R, Damon/IEC).
4. The supernatants were poured off gently, the pellets were resuspended by mild agitation, and the tubes were topped up with PBS and washed as in step 3.
5. Again the supernatants were poured off gently, but this time the pellets were not resuspended.
6. To each pellet was added 100µl of GAM-FITC (FITC-conjugated goat anti-mouse antibodies; previously diluted 1:10 in PBS to give a working concentration). The contents were mixed and placed on ice for 30min with regular mixing.
7. The cells were washed twice as in steps 3, 4, and 5.
8. For flow cytometric analyses, the cell pellets were gently resuspended in 1ml of Sheath Fluid (Becton Dickinson).

Appropriate isotype controls were set up for each antibody :- mouse IgG_1(Serotec; MCA 1209) and mouse IgG_2(Serotec; MCA 1210).

2.21 Cytogenetic analyses of REL-C7 cells

Cytogenetic analyses were kindly performed by Dr. George Breckon, MRC Radiobiology Unit, Chilton, Didcot, Oxon OX11 0RD, UK (presently retired). Briefly, REL-C7 cells were maintained under standard conditions in MEM-O++.

1. To arrest proliferation and allow the cells to accumulate in mitosis, colcemid (Sigma) was added to a final concentration of 0.05μg/ml (10μl of 50μg/ml stock colcemid into 10ml of culture). The flask was incubated for 10min at 37°C.
2. The contents of the flask were transferred to a sterile, 15ml centrifuge tube (Costar) and spun down at 200g for 10min. The supernatant was carefully poured off and the cells resuspended in the residual supernatant by gently tapping the tube.
3. The cells were then subjected to hypotonic treatment. This causes the cells to swell and become fragile, so that when they are dropped onto slides, the cells burst, spreading out their chromosomes for inspection. The hypotonic agent was 0.075M KCl (BDH). Ten millilitres of KCl was added to the resuspended cells, which were then incubated for 10min.
4. The cells were gently centrifuged at 100g for 10min. The supernatant was carefully poured off and the cells resuspended by gentle agitation in the residual supernatant.
5. The cells were next fixed by the addition of 10ml of freshly prepared fixative (absolute methanol : glacial acetic acid, 3 : 1) (AnalaR grade, BDH).
6. The cells were spun at 100g for 10min and the fixative replaced with fresh fixative.
7. Slides were thoroughly cleaned with absolute alcohol. From about 2 - 5 inches above the slide, 2- 3 drops of cell suspension were dropped onto the slide. The slides were allowed to air-dry and were G-banded by a modification of the method described by Gallimore and Richardson (172).

8. The slides were transferred to a rack and placed in 2X SSC (0.3M NaCl, 0.03MNa Citrate, pH 7.2) for 2hr at 60°C.

9. The slides were then taken through successive solutions of saline; distilled water; 50% methanol; 70% methanol; 90% methanol; and absolute methanol (1 min in each). The slides were then air-dried, prior to trypsinization.

10. Trypsin solution was prepared at 0.125% (Life Technologies) in saline (1 vial of 20ml of 2.5% trypsin, made up to 400ml with saline). The slides were placed in the trypsin-saline solution for 20 - 30 secs.

11. Finally, the slides were washed in de-ionized water and stained for 2 - 3 min in Giemsa stain (Gurr's R66) (BDH, Catalogue number 35086) (Giemsa at 10% in pH 6.8 buffer). The stained preparations were air-dried and mounted in Depex (BDH, Catalogue number 36125).

12. Photographs were taken on Kodak Technical Pan 2145 film. Thirty metaphases were analyzed under light microscopy and a further 20 metaphases were photographed and karyotyped.

SECTION 3

RESULTS

3.1 Growth Characteristics of REL-C7 in Liquid Culture

In most cell culture procedures, serum is used to supplement the basic culture medium. Often, the batch of serum used is of crucial importance. To ensure, as far as possible, that the cells were being maintained under optimal conditions, batches of serum were pretested for their suitability. In addition, REL-C7 cells were cultured in medium containing a range of FBS concentrations to define further the optimum conditions.

3.1.1 Choice of FBS

Originally, a batch of FBS was used which was already available in the laboratories, used in another unrelated culture activity (Flow Laboratories, Batch 29124125).

The following batches of FBS were tested:-

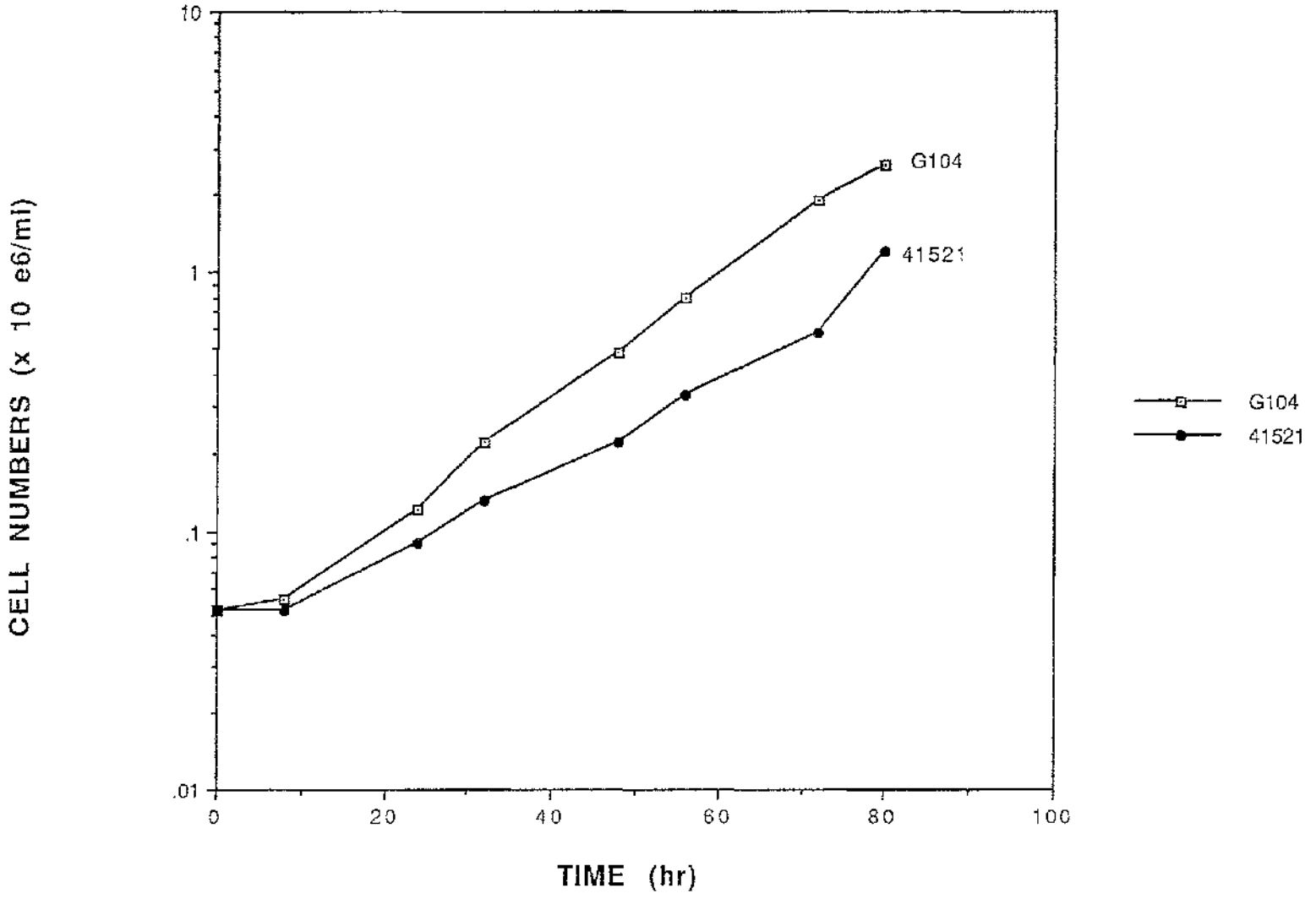
Source	Batch No.
Globepharm	G104
Gibco/BRL	10Q2152A
Advanced Protein Products	SBS70
Northumbria Biologicals	41521
Gibco/BRL	40Q

Aliquots of MEO-O± were supplemented with one of the above sera to give multiple batches of MEM-O+.

REL-C7 cells were inoculated into 25 cm^2 flasks each containing 5 ml of one of the media. As outlined in Section 2.3, cultures were maintained for one week to allow the cells to adapt fully to the change in FBS.

On (an arbitrary) day 0 (d.0), fresh cultures were established in each of the media with the starting concentration at 1.0×10^4/ml. At daily intervals, a small aliquot of culture was aspirated and counted (Section 2.15). These data were plotted

Fig.3
Growth curve of REL-C7 cells cultured with various batches of FBS



as cell concentration versus time. From the logarithmic phase of the growth curve, cell doubling times were calculated. A representative example is shown in Fig 3.(comparing batch G104 with batch 41251). In addition, the cells were assessed for viability by Trypan Blue exclusion (Section 2.4.1)

With the notable exception of batch 41521, there was no significant difference between the batches of FBS tested (Table 1). Apart from batch 41521, the doubling times were consistently between 12-14hr with high viability (Table 2). Unless otherwise indicated, batch G104 was used for the subsequent investigations.

3.1.2 Effect of various FBS concentrations on REL-C7 growth

To define further the optimal conditions, media were prepared with a range of concentrations of FBS:- 1%, 2.5%, 5%, 10%, 15%, 20%, 30%. As before, cells were allowed to adapt to the different FBS concentrations. Daily cell counts and viability were performed. A representative example is illustrated in Fig 4.

A clear pattern was observed in the growth characteristics. The optimal range of FBS concentrations was between 5% and 20% with no significant difference in the doubling times at >5% FBS (Table 3). At high FBS concentration (30%) the doubling time was apparently longer, though not significantly so. Below 5% FBS, the doubling times became progressively longer at 2.5% FBS and 1% FBS. A similar pattern was observed with the cell viabilities (Table 4). At FBS concentrations between 5% and 30% viabilities were consistently and invariably high (>96%). At lower concentrations, particularly 1% FBS, there was a tendency for viability to decrease over the time-course of the experiment so that by day 4 of culture, the viability was down to 82%.

A broad range of FBS concentrations (5%-30%) seemed able to maintain REL-C7 growth, although at 30% FBS there was some evidence to indicate that this concentration was slightly inhibitory as demonstrated by the extended doubling time. Lower concentrations (1% and 2.5%) were clearly sub-optimal, with the doubling times extended to 23 hr and 28 hr respectively, with a concommitant decrease in cell

Table 1

Effect of various batches of FBS on REL-C7 growth.

Data expressed as mean ± SD of three experiments.

FBS Batch	Doubling Time (hr)
29124125	12.7 ± 0.35
G 104	12.7 ± 1.1
10 Q	13.0 ± 0.61
SBS 70	13.4 ± 1.0
41521	17.7 ± 1.4
40 Q	13.1 ± 0.7

Table 2

Effect of various batches of FBS on REL-C7 viability.

Data expressed as mean ± SD of three experiments.

FBS Batch	Day 0	Day 1	Day 2	Day 3	Day 4
29124125	97.0 ± 1.0	98.3 ± 0.6	98.3 ± 1.1	97.7 ± 1.5	97.3 ± 0.6
G104	98.0 ± 1.0	97.7 ± 0.6	98.0 ± 1.7	97.0 ± 1.0	97.7 ± 5.3
10 Q	97.3 ± 1.1	96.7 ± 1.5	97.7 ± 2.2	97.7 ± 1.5	97.0 ± 1.0
SBS 70	97.0 ± 1.7	96.9 ± 1.1	98.3 ± 1.1	98.7 ± 0.5	98.7 ± 0.6
41521	87.0 ± 3.6	88.0 ± 4.6	92.0 ± 3.0	88.3 ± 3.0	92.7 ± 5.1
40 Q	97.3 ± 1.2	98.3 ± 1.2	98.0 ± 1.0	98.3 ± 0.6	97.7 ± 0.6

Fig.4
Growth curves of REL-C7 cultured with various concentrations of FBS.

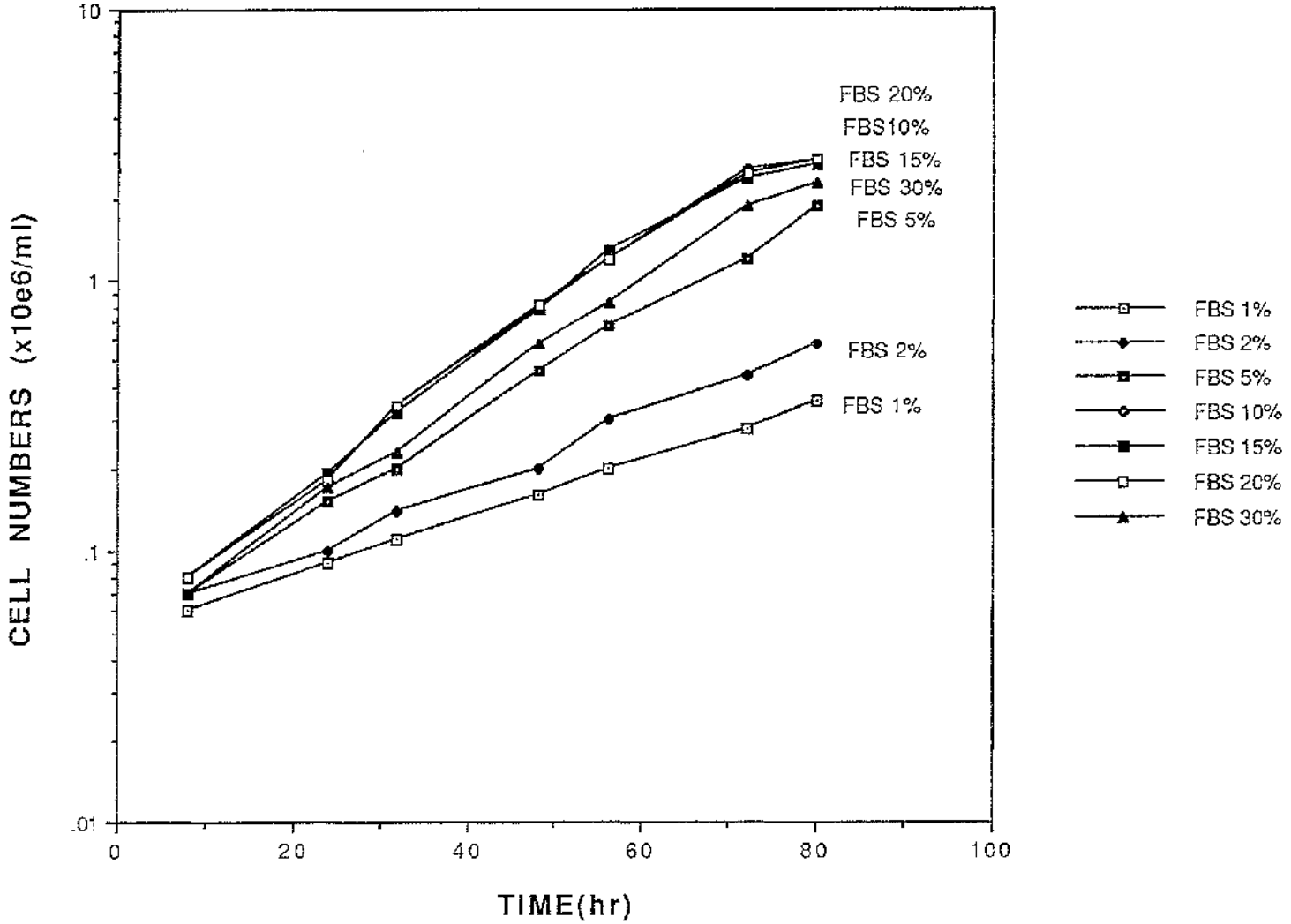


Table 3

Effect of various FBS concentrations on REL-C7 growth.

Data expressed as the mean ± SD of three experiments.

FBS Concentration (%)	Doubling Time (hr)
1.0%	28.0 ± 3.6
2.5%	22.9 ± 1.7
5.0%	13.6 ± 0.7
10%	12.6 ± 0.4
15%	13.4 ± 0.6
20%	12.8 ± 0.5
30%	14.1 ± 0.4

Table 4

Effect of various FBS concentrations on REL-C7 viability.

Data expressed as the mean ± SD of three experiments.

[FBS] (%)	Day 0	Day 1	Day 2	Day 3	Day 4
1.0%	96.7±+ 0.6	97.0 ± 1.7	97.7 ± 0.6	91.7 ± 1.5	82.0 ± 3.0
2.5%	98.0 ± 1.7	98.3 ± 1.1	97.0 ± 1.0	90.3 ± 1.5	91.7 ± 2.1
5.0%	99.0 ± 0.6	96.0 ± 2.0	98.3 ± 1.1	98.3 ± 0.6	99.0 ± 0
10%	98.3 ± 1.7	98.7 ± 0.5	98.7 ± 0.6	98.7 ± 0.6	97.7 ± 0.6
15%	98.3 ± 0.6	98.0 ± 1.7	98.0 ± 1.2	98.3 ± 0.6	97.7 ± 2.2
20%	98.0 ± 1.0	98.3 ± 1.1	99.0 ± 0	99.0 ± 0	97.7 ± 0.6
30%	98.0 ± 1.0	98.7 ± 0.6	98.3 ± 0.6	98.7 ± 0.5	98.3 ± 0.6

viability. For routine maintenance, 10% was deemed to be the most appropriate concentration of FBS.

3.2 Clonogenic assay for REL-C7 cells

Liquid culture conditions allow the proliferation of a population of cells to be monitored by performing cell counts over a period of time. In many experiments, however, it can be much more informative to look at the behaviour of individual cells within a population. Clearly, liquid culture does not allow such single cell analyses.

The behaviour of individual cells was assessed in a clonogenic, semi-solid assay system (Section 2.5). In this culture system, cells were immobilised in a viscous medium (MEM-O++/methyl cellulose). In a given population of cells, normally only a proportion of the total number will proliferate and form discrete clones. The cloning efficiency of a particular cell line may be calculated as :-

$$\frac{\text{Total no.of colonies counted}}{\text{Total no.of cells plated}} \times 100\%$$

The cloning efficiency of REL-C7 cells was calculated in a series of experiments by culturing REL-C7's at various concentrations. Cells were routinely maintained in liquid culture (see Section 2.3) at a cell concentration between 5×10^4/ml and 2×10^6/ml. Exponentially growing cells were aspirated from stock cultures and transferred to a 15 ml centrifuge tube. Cells were spun down at 100 g for 10min. Cells were resuspended in a small (<1ml) volume of fresh MEM-O++ and a sample was removed for counting. Appropriate dilutions were made to allow a range of final cell concentrations to be set up in the clonogenic assay.

Example : REL-C7 cells resuspended at 4.0×10^6/ml.

The cell suspension was diluted 100 - fold:-

100 µl of 4.0×10^6/ml
9.9 ml of MEM-O++
} 10ml of 4×10^4/ml

Serial 10 -fold dilutions were performed:-

100 µl of 4 x 10^4/ml
900 µl of MEM-O++ } 1 ml of 4 x 10^3/ml

100 µl of 4 x 10^3/ml
900 µl of MEM-O++ } 1 ml of 4 x 10^2/ml

100 µl of 4 x 10^2/ml
900 µl of MEM-O++ } 1 ml of 4 x 10^1/ml

Three tubes of 1.8 ml methylcellulose/FBS were prepared.

Tube 1 : received 600 µl of 4 x 10^3/ml
Final concentration : 1 x 10^3/ml

Tube 2 : received 600 µl of 4 x 10^2/ml
Final conentration : 1 x 10^2/ml

Tube 3 : received 600 µl of 4 x 10^1/ml
Final concentration : 1 x 10^1/ml

Cells were plated out as described in Methods (Section 2.5.2), and colonies counted after 4 days incubation (Table 5). A colony characteristically appeared as a fairly compact, "ball" of cells (Plate 1).

Over a range of plating concentrations, REL-C7 showed a remarkable consistency in cloning efficiency of between 55 and 59%. Dilution effects/low cell numbers contributed to the imprecision of the experiments at 1 x 10^2/ml and 1 x 10^1/ml. The most reproducible cell concentration to plate out in the clonogenic assay

Table 5

Cloning efficiency of REL -C7.

No. of colonies counted

No.of cells plated	Exp.1	Exp.2	Exp.3	Exp.4	Exp.5	Exp.6	Mean ± SD	Range (%)
1×10^3	527	610	582	683	587	554	59 ± 7.3	53 - 68
1×10^2	40	68	55	71	61	49	57 ± 34.3	40 - 71
1×10^1	4	6	6	5	7	5	55 ± 10.0	40 - 70

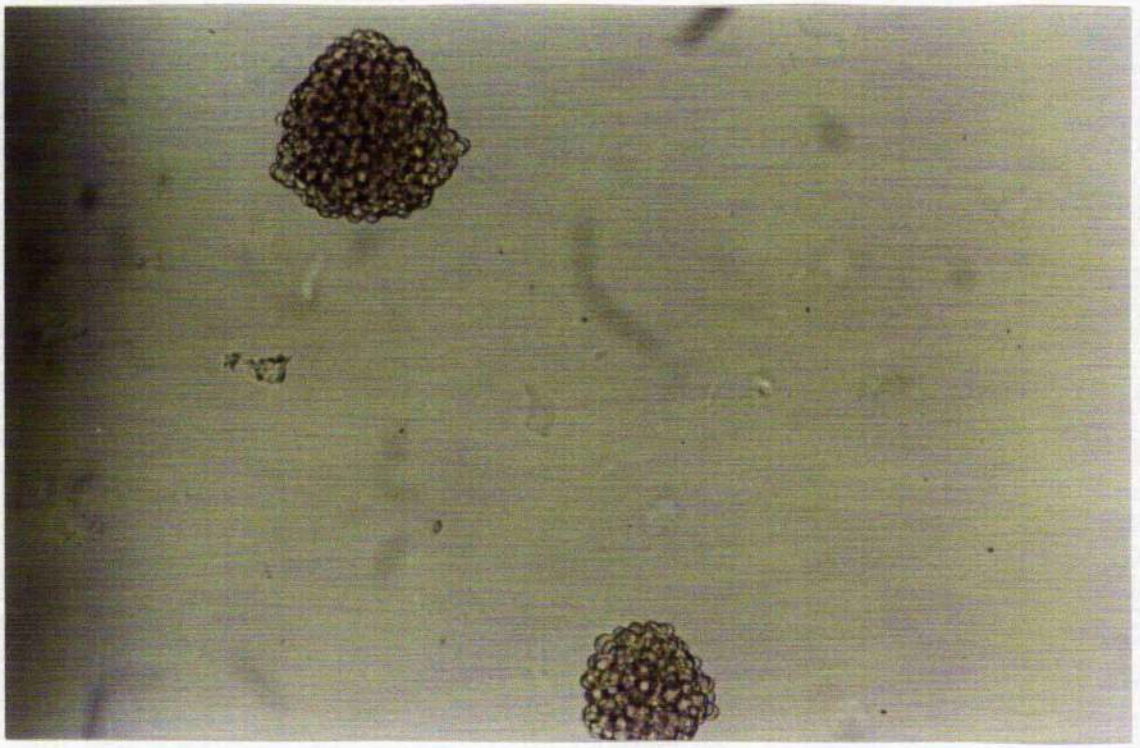
Plate 1

REL-C7 cells grown in methylcellulose culture.

Cell concentration 1 x 10^3/ml.

Cultured for 4 days

Magnification 400X.



was 1 x 10^3/ml. This gave a mean cloning efficiency of 59%. In initial experiments, cells were plated at concentrations greater than 1 x 10^3/ml, e.g. 5 x 10^3/ml and 1 x 10^4/ml. However, these cultures produced so many colonies that the plates could not be counted due to colonies overlapping.

Another aspect of this clonogenic assay was its "sensitivity", i.e. when very low numbers of cells (e.g. 10 cells) were plated out, the pattern of colony formation was remarkably similar to the ideal plating concentration (i.e. 1000 cells/ml). This assay, therefore, represents a very sensitive means of detecting proliferating REL-C7 cells with clonogenic potential. This characteristic becomes important when REL-C7 differentiation is analyzed, i.e. as cells become committed to differentiation they lose their proliferative ability. This may be monitored at the single cell level by a clonogenic assay.

3.3 *in vivo* assay for REL-C7 (Tumourogenicity)

The malignant nature of REL-C7 cells can be demonstrated in an *in vivo* tumourogenic assay (see Section 2.6).

Series of experiments were set up to investigate how the numbers of cells injected per animal influenced the time taken to tumour formation. In addition, low cell concentrations were tested to determine the minimum number of cells required to induce tumour formation, i.e. "sensitivity".

Long Evans babes, between 3 and 7 days old, received a subcutaneous injection of 0.1ml containing a range of REL-C7 numbers. To ensure that the physical trauma of a subcutaneous injection had no effect, control animals received an injection of 0.1ml of culture medium (MEM-OH$^-$) containing no cells. The animals were observed regularly (e.g. every two days) for the appearance of a tumour at the site of injection (see Table 6). On the day of detection, tumour diameter would be approximately 5mm and certainly no greater than 10mm. As soon as tumour formation was confirmed, the animals were killed. It was noted that, if animals were allowed to survive beyond this point, the tumour cells continued to proliferate (Plate

Table 6
Incidence of tumour formation.

Cells injected per babe	Day 0	Day 5	Day 7	Day 9	Day 11	Day 13	Day 15	Day 17	Day 19	Day 21	Day 23	Day 25	Overall Incidence (%)
1×10^5	0 / 15	0 / 15	10 / 15	15 / 15									100
1×10^4	0 / 23	0 / 23	5 / 23	10 / 23	17 / 23	22 / 23	23 / 23						100
1×10^3	0 / 47	0 / 47	0 / 47	0 / 47	0 / 47	2 / 47	8 / 47	16 / 47	27 / 47	38 / 47	47 / 47		100
1×10^2	0 / 24	0 / 24	0 / 24	0 / 24	0 / 24	0 / 24	3 / 24	12 / 24	21 / 24	22 / 24	22 / 24	23 / 24 →d35	96
1×10^1	0 / 22	0 / 22	0 / 22	0 / 22	0 / 22	0 / 22	0 / 22	0 / 22	3 / 22	9 / 22	9 / 22	10 / 22 →d39	45
0	0 / 15	0 / 15	0 / 15	0 / 15	0 / 15	0 / 15	0 / 15	0 / 15	0 / 15	0 / 15	0 / 15	0 / 15	0

Plate 2

Long Evans babes showing tumour formation.

Animals each received a cell dose of 1 x 10^4 REL-C7 cells.

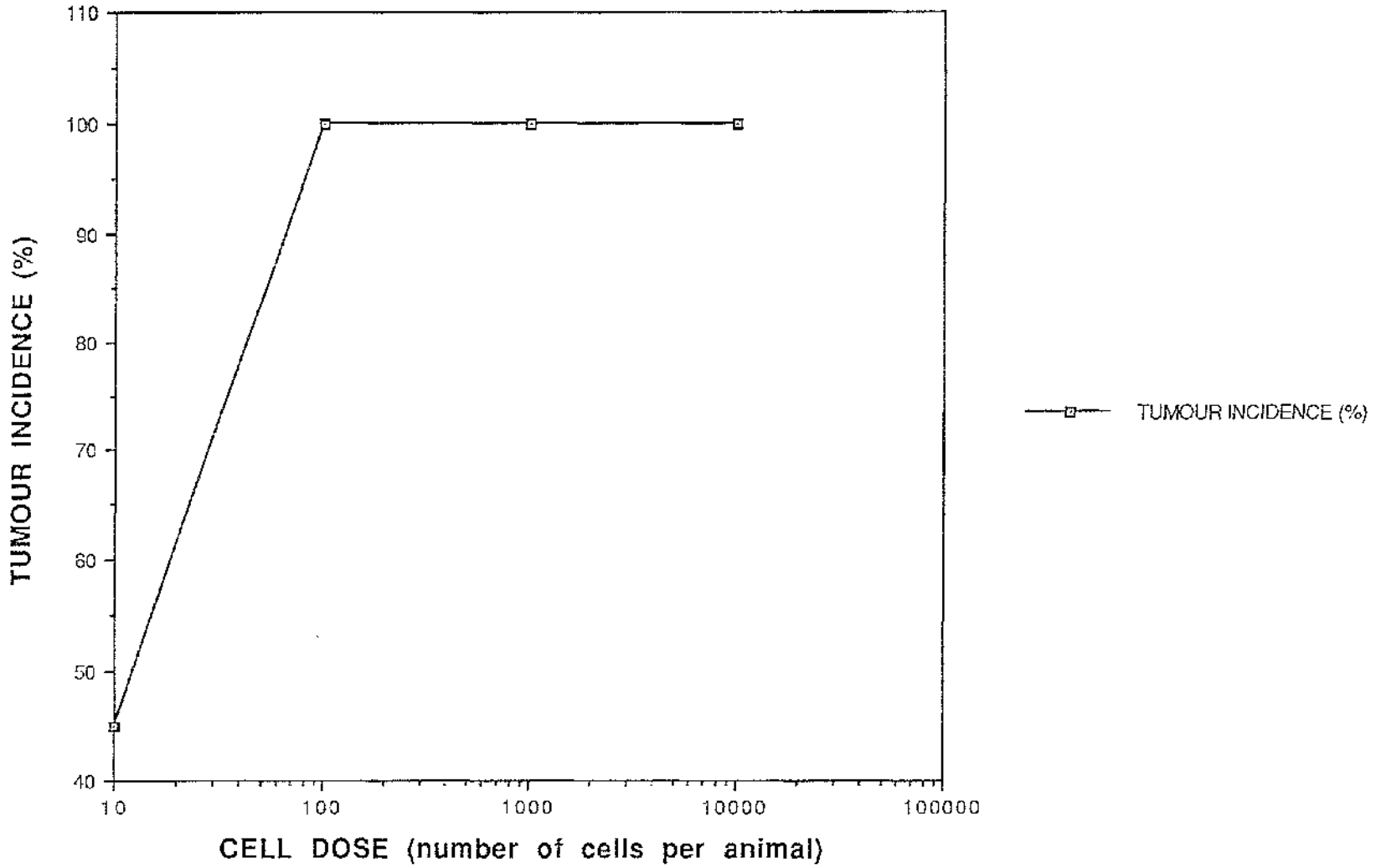
Assessment shown at day 12.

2) until a massive dorsal growth developed.

Clearly, there is a dose response between the number of cells administered and the incidence of tumour formation. There is also a relationship between the number of cells administered and the time to tumour development. Tumour incidence was 100% in the group that received either 1 x 10^5cells/babe, 1 x 10^4cells/babe or 1 x 10^3cells/babe. In the group that received 1 x 10^2cells/babe, tumour incidence was 96% (i.e. 1 animal remained tumour free). In the group that received 1 x 10^1cells/babe, tumour incidence was 45% with only 10 out of 22 animals developing a tumour. The TD_{50} was calculated i.e. TD_{50} = tumour dose 50 i.e. the dose (number) of cells required to elicit tumour development in 50% of the recipient animals (see Figure 5). The TD_{50} was 1.2 x 10^1cells.

In the larger cell inocula (1 x 10^5/babe, 1 x 10^4/babe) tumour development was rapid with the first tumours being detected by d.7. Indeed in the 1 x 10^5 group, all 15 animals had been sacrificed by d.7. In the 1 x 10^4 group, overall tumour development was slower with progressively more animals developing tumour until d.15 by which time all were positive. The same general pattern was observed in the 1 x 10^3 group, although tumour development was delayed until d.13. Again, tumour incidence progressively increased until all animals were positive by d.23. When the number of cells injected was reduced to 1 x 10^2/babe, no tumour was detected until d.15. Animals continued to develop tumour up until d.25 by which time 23 of 24 (96%) were positive. Due to the protracted time course of tumour development, the animals in this group were observed up to d.35 in case any later developing tumours appeared. It was felt that after this time, there was little likelihood of any further tumours appearing. In the group of animals receiving 1 x 10^1 cells/babe, no tumour was detected until d.19. Subsequently, a total of 10 of 22 (45%) animals developed tumour. Again, because of the prolonged period of time taken for any tumour development, these animals were observed up to d.39, however no further tumours developed after d.24. As expected, no tumours were detected in the control group.

Fig.5
TD50 for L.E. babes injected with various concentrations of REL-C7.



Clearly, the time course of tumour development is a "numbers game", i.e. (up to a point) the more cells you add, the quicker the tumour develops. The end-point of this assay is rather crude and subjective - namely, on what day did the observer detect a tumour. By necessity, this measurement is somewhat imprecise. Nevertheless, it would be anticipated that an inoculum of 1×10^5 cells would form the critical mass of cells that is detectable as tumour more quickly than, say, 1×10^1 cells. If tumour formation simply reflected the number of cells injected, then one might expect that, in a particular group, tumour would appear on all the animals on, more of less, the same day. Clearly, this is not the case. For example, in the group that received 1×10^3 cells/babe, although no tumour was detected until d.13, animals continued to develop tumour over the ensuing 10 days. Similarly, in the group that received 1×10^2 cells/babe, tumour was detected from d.15 through to d.25. This imprecision in the time taken for tumour formation within a particular group can be explained, at least in part, by natural variation between animals. These data were accumulated from 20 litters of L.E.babes. One would expect differences, certainly, between litters and probably some variation within a litter as well. Taking into account this population heterogeneity, one would expect considerable variation in the ease with which injected cells could produce a tumour.

There is also an inherent inaccuracy in the number of cells administered per animal. First of all, every time the cells are diluted another inaccuracy is introduced. For the animals who received 1×10^1 cells, the cell concentration was "1×10^2/ml". This can only be regarded as a nominal value. Careful laboratory technique can minimise this effect, but cannot overcome it. Similarly the cell inoculum was a volume of 0.1 ml using a 1 ml syringe. Although great care was taken to administer 0.1ml, there was bound to be significant aliquot to aliquot variation. Another source of inaccuracy was occasionally observed with the actual injection procedure. After injection of the cells, as the needle was withdrawn, it was not uncommon for a small drop of the cell suspension to leak out of the puncture hole. Obviously, such animals did not receive the planned dose of cells.

Notwithstanding these limitations, the tumourogenic assay still represents a convenient and sensitive means of measuring a fundamental property of the REL-C7 cell line, namely, its malignancy. In later sections, data will be presented showing how the growth characteristics of these cells can be modulated and how these alterations affect the malignant nature of the cells.

3.4 Chemically - induced erythroid differentiation of REL-C7

It has been known for some years that cell lines established from 7,12-DMBA induced leukaemia can be stimulated to differentiate along an erythroid pathway (143). On exposure to, for example, DMSO or HMBA, cells can be induced to differentiate and to synthesize adult rat haemoglobin. Although the original leukaemia and subsequent leukaemic cell lines appear morphologically relatively undifferentiated, the cells are generally regarded as erythroleukaemic (REL-cells: Rat Erythroleukaemic).

3.4.1 DMSO - induced differentiation

Experiments were set up to establish the optimum conditions for erythroid differentiation of REL-C7 by DMSO. Cultures of REL-C7 cells were exposed to a range of concentrations of DMSO and the extent of differentiation was assessed daily by the benzidine stain for the detection of haemoglobin (see Section 2.7 Materials and Methods). In addition, morphological assessment was also carried out. Plate 3 shows a typical benzidine stain. Those cells that have accumulated haemoglobin, stain with a strong blue colouration. Those cells which have not differentiated, do not produce haemoglobin and appear yellow.

Morphological analyses also demonstrated the fundamental changes that take place in cells exposed to DMSO. REL-C7 cells maintained in MEM-O++ under standard culture conditions, had a primitive appearance (Plate 4A) with Wright's stain. The nucleus usually had a finely reticular appearance and nucleoli could be discerned. The cytoplasm was basophilic (deep blue in colour). They could probably be classed as (pro?)erythroblasts.

Plate 3

Benzidine staining of REL-C7 cells exposed to 1.5% DMSO for 4 days.

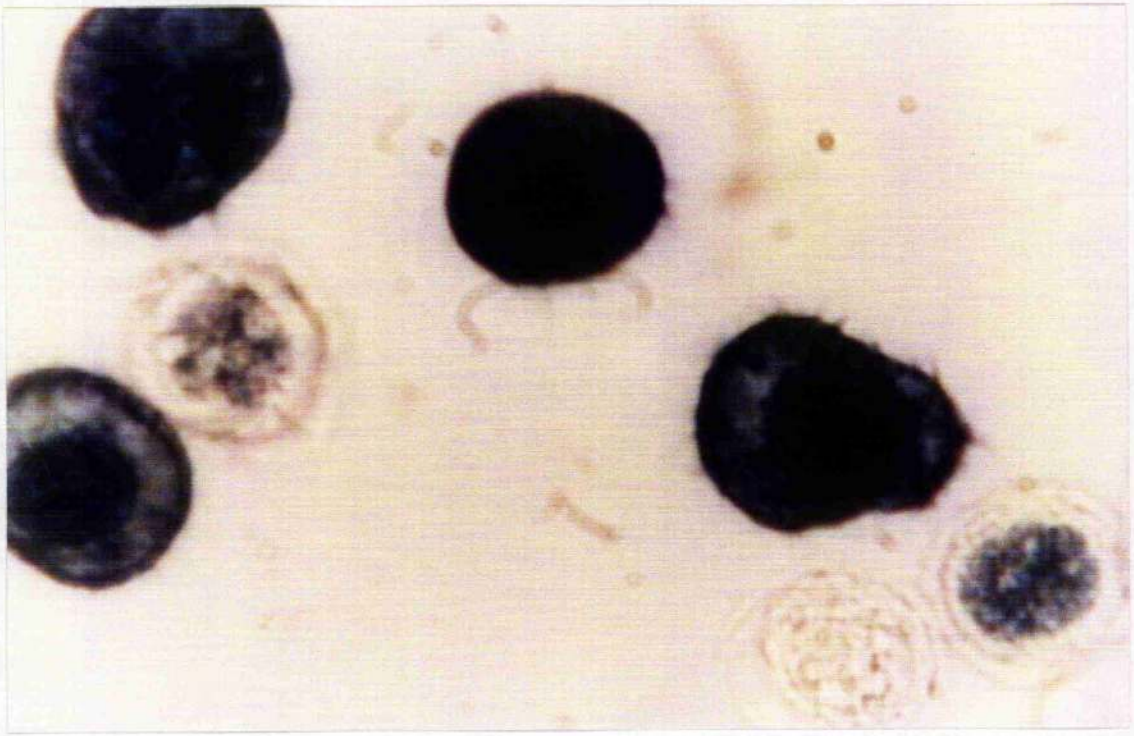
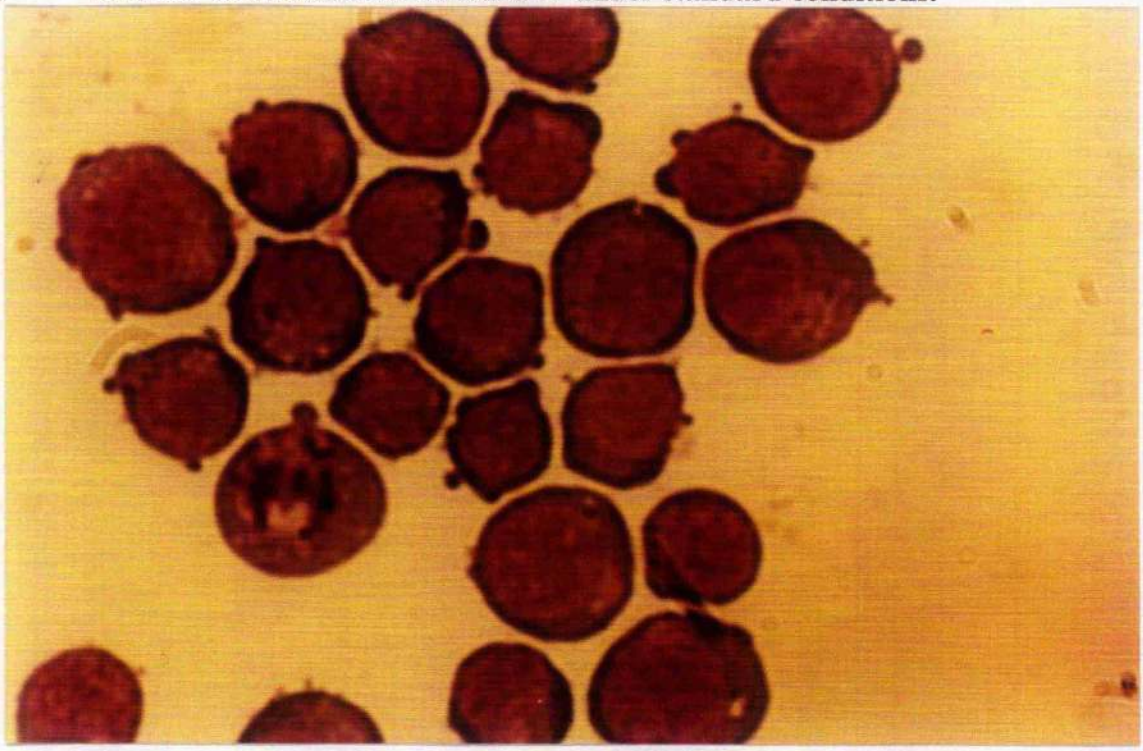


Plate 4

Morphology of REL-C7 cells by Wright stain.

Magnification 400X.

A. REL-C7 cells culture in MEM-O++ under standard conditions.



B. REL-C7 cells cultured in MEM-O++, incubated with 1.5% DMSO for 4 days

Following exposure to DMSO, it could be readily seen that these cells had matured to a clearly recognizable erythroid morphology (Plate 4B). As the cells differentiated, there was a marked reduction in cell volume and the nucleus : cytoplasm ratio decreased. The nucleus was often eccentric, with clumped chromatin. Haemoglobin production was indicated by the pink perinuclear colouration in the cytoplasm. In some cases, cells matured even further and a condensed, pyknotic nucleus could be observed in a rather featureless, pink cytoplasm. These cells resembled erythroid cells at the late normoblast stage of development. In the occasional cell, the nucleus seemed to be in the process of extrusion, i.e. oneof the final stages in mature red blood cell production.

Data from 4 experiments are summarised in Table 7.

Several conclusions can be drawn:-

a) Irrespective of the concentration of DMSO used, significant haemoglobinization is not detectable until 72 hr of culture. Certainly, up to a DMSO concentration of 0.5% there was no haemoglobinization at all over the first 48 hr. DMSO concentrations between 0.75% and 2.0% did produce some benzidine positive cells after 48 hr culture, but these numbers were very low, never constituting more than 2% in any one experiment. It was only after 72 hr incubation that high levels of benzidine positive cells could be detected reproducibly. At the higher concentrations of DMSO (1.0% - 2.0%) a dramatic increase in benzidine positive cells was demonstrated between 48 and 72 hr of culture, and this increase continued, although less markedly, up to 96 hr of culture.

There is a low level of spontaneous haemoglobinization which occurs in the absence of DMSO. When REL-C7 cells are cultured in MEM-O++ under standard conditions, occasional benzidine positive cells may be observed. In any one flask these spontaneously occurring benzidine positive cells rarely exceeded 1%. A typical result would be that, if 400 cells were counted, 1 or 2 might be benzidine positive, i.e. < 1%. Additional data are reported in Section 3.5.1.

Table 7

Effect of various concentrations of DMSO on erythroid differentiation of REL-C7.

Percentage of cells staining positive for the benzidine reaction.

Cells were cultured for 96hr in MEM-O++ supplemented with the appropriate volume of DMSO.

Data are expressed as the mean ± SD of four experiments.

Concentration of DMSO (v/v)	0 hr	24 hr	48 hr	72 hr	96 hr	120 hr
0	<1	<1	<1	<1	<1	<1
0.10%	<1	<1	<1	1	1	<1
0.25%	<1	<1	<1	3.25 ± 1.3	5.25 ± 1.7	2.25 ± 1.3
0.50%	<1	<1	<1	3.75 ± 2.4	7.50 ± 1.7	3.50 ± 2.6
0.75%	<1	<1	1	8.25 ± 1.3	12.3 ± 2.5	9.0 ± 1.8
1.0%	<1	<1	1	16.5 ± 3.9	20.5 ± 5.0	17.8 ± 3.9
1.25%	<1	<1	1	27.0 ± 6.3	45.8 ± 6.2	31.3 ± 11
1.50%	<1	<1	1.5 ± 0.6	45.8 ± 6.2	64.0 ± 5.0	ND
1.75%	<1	<1	1.5 ± 0.6	30.5 ± 4.7	54.5 ± 6.6	ND
2.0%	<1	<1	1.25 ± 0.5	21.0 ± 3.7	31.0 ± 8.6	ND

ND = Not Done (cells unevaluable).

b) The lowest concentration of DMSO that produced appreciable haemoglobinization was 0.25%. At this concentration only a moderate level of erythroid differentiation was obtained with a peak value of 5.25% on day 4, which subsequently declined to 2.25% following a further 24 hr of incubation. A similar pattern was observed when DMSO was present at 0.5%.

c) The concentration of DMSO that produced the greatest degree of haemoglobinization was 1.5%. The first increase in benzidine positive cells was detected after 48hr incubation, but only at a modest level of 1.5%. Over the next 24 hr the numbers rose rapidly from 1.5% to 45.8% and increased further, though not to the same degree, over the next 24 hr to 64.0%. The general trend was that as the DMSO concentration increased, then so too did the percentage of benzidine positive cells. This relationship was observed up to the optimum DMSO concentration of 1.5%. DMSO concentrations in excess of this (i.e. 1.75%, and 2.0%), showed decreased benzidine positivity.

d) For each individual DMSO concentration studied, the maximum number of benzidine positive cells was obtained after 96 hr culture. Beyond this time point, there was a reduction in the number of erythroid differentiated cells when the DMSO concentration was sub-optimal. At the lower DMSO concentrations, e.g. DMSO 0.25% and 0.50%, only a relatively small proportion of cells had been induced to differentiate, as demonstrated by the low percentage benzidine positive results. It appeared, therefore that those cells that had not been committed to differentiate, continued to proliferate. By 120 hr of culture, these proliferating cells had proliferated sufficiently to reduce the relative number of benzidine positive cells. A different phenomenon was observed at higher DMSO concentrations. After 120 h of culture, with DMSO at either 1.75% or 2.0%, the cells were difficult to evaluate. A marked deterioration in the appearance of the cells was observed, e.g. ple omorphic cells debris. Trypan blue assessment of these cultures revealed considerable cell damage (Table 8). Reliable analyses could not be performed. The other DMSO concentrations between these two extremes (i.e. say, 1.0% - 1.5%) also showed some

Table 8

Trypan Blue viability assessment of REL-C7 cells exposed to various DMSO concentrations.

% DMSO concentration	0 hr	72 hr	96 hr	120 hr
1.0 %	99	99	97	98
1.25 %	99	98	97	95
1.50 %	99	97	98	96
1.75 %	99	96	93	90
2.0 %	99	98	85	83

deterioration in the quality of the cells. But, at the same time, a significant proportion of undifferentiated cells continued to proliferate.

The optimum concentration of DMSO to produce the maximum number of benzidine positive cells was deemed to be 1.5%. Below this concentration differentiation was sub-optimal. Above this concentration excessive cell death occurred.

3.4.2 Effect of various compounds on REL-C7 differentiation

Although DMSO is the compound most widely used to induce differentiation in REL cells, other chemicals have been identified which can induce differentiation in similar cell lines (e.g. murine Friend erythroleukaemic cells). Experiments were set up to investigate these compounds to see if they were effective in inducing differentiation in REL-C7.

3.4.2.1. HMBA (hexamethylenebisacetamide)

Stock HMBA was prepared as outlined in Section 2.7.2 at a concentration of 50 mM. Cultures of REL-C7 cells were exposed to a range of concentrations of HMBA and the extent of differentiation was assessed, as before, by the benzidine reaction. As with all investigations of putative inducers, flasks of medium were pre-equilibrated, had the appropriate volume of stock chemical added, and only then were cells inoculated into the flasks. Data from 4 experiments are summarised in Table 9.

The results show some similarities to DMSO-induced differentiation. As with DMSO, significant levels of haemoglobinized cells were first detected after 72 hr of culture, regardless of the HMBA concentration. At any one concentration of HMBA, maximal haemoglobinization was always obtained after 96 hr of culture, the levels thereafter declining. The concentration of HMBA that was most effective in inducing differentiation was in the range 2mM - 3mM. HMBA at 3 mM seemed slightly superior, but not significantly so (Students t-test, $p>0.5$). Although HMBA could reproducibly induce REL-C7 cells to differentiate, it was less effective than

Table 9

Effect of various concentrations of HMBA on erythroid differentiation of REL-C7.

Percentage cells staining positive for the benzidine reaction.

Data are expressed as the mean ± SD of four experiments.

[HMBA] mM	0 hr	24 hr	48 hr	72 hr	96 hr	120 hr
0	<1	<1	<1	<1	<1	<1
1.0 mM	<1	<1	1.0	4.3 ± 3.1	6.3 ± 5.0	1.0
2.0mM	<1	<1	1.0	10.5 ± 5.9	24.0 ± 13	10.0 ± 1.6
3.0 mM	<1	<1	1.0	13.0 ± 6.6	27.8 ± 11	25.5 ± 3.6
4.0 mM	<1	<1	1.5 ± 0.6	3.3 ± 4.8	9.5 ± 9.6	NE

NE = Not Evaluable

DMSO. The highest mean level of haemoglobinization was 27.8% when cells were cultured for 96 h in the presence of 3mM HMBA. In one of the four experiments, at this concentration, 40% benzidine positivity was obtained. This was the highest result recorded using HMBA.

In summary, HMBA can induce REL-C7 cells to differentiate in similar fashion to DMSO. However, HMBA is inferior to DMSO as an inducer of erythroid differentiation.

3.4.2.2 ATRA (all trans Retinoic Acid)

Stock ATRA was prepared as outlined in section 2.7.3. at a concentration of 100 mM. Since ATRA requires to be dissolved in ethanol, a preliminary experiment was set up to assess the effect of ethanol alone on REL-C7 cells (Table 10). Ethanol had surprisingly little effect on the growth and viability of REL-C7 cells, even at concentrations as high as 0.5% (v/v) (i.e. 25 µl ethanol per 5 ml culture). Over the range of ethanol concentrations studied, there was no appreciable difference in cell numbers or viability.

A range of concentrations of ATRA was prepared. To minimise any potential effects of the ethanol, ATRA was added in a volume of 5 µl per 5 ml of culture, i.e. 1:1000. Consequently, all the ATRA concentrations had to be prepared at 1000 - fold the desired final concentration; e.g. to obtain a final ATRA concentration of 10^{-5} M, 5 µl of ATRA at 10^{-2}M were added to 5 ml medium. As usual, the appropriate volume of cells was added last. Data from 2 experiments are summarised in Table 11.

From the data it can be seen that ATRA can indeed induce erythroid differentiation in REL-C7 cells. In comparison to cells induced by DMSO (or HMBA), with ATRA the appearance of haemoglobinized cells was somewhat delayed until 72 h of culture (cf. 48 hr for DMSO). The concentration of ATRA that produced the highest percentage of benzidine positive cells was 1 x 10^{-5}M. This concentration gave a rather modest 18.5% after 96 h culture. A concentration of 1 x 10^{-6}M seemed

Table 10

Effect of various ethanol concentrations on growth of REL-C7.

In all cases, the viability was ≥ 96%.

Data are cell numbers x10^6/ml.

[Ethanol] (%)	0 hr	24 hr	48 hr
0	0.10	0.29	0.89
0.001	0.10	0.28	0.79
0.005	0.10	0.25	0.63
0.010	0.10	0.22	0.76
0.050	0.10	0.26	0.88
0.10	0.10	0.23	0.79
0.50	0.10	0.27	0.71

Table 11

Effect of various ATRA concentrations on erythroid differentiation of REL-C7.

Percentage of cells staining positive for the benzidine reaction.

[ATRA](M)	0 hr	24 hr	48 hr	72 hr	96 hr
0	<1	<1	<1	<1	<1
10^{-8}	<1	<1	<1	2.0	2.5
10^{-7}	< 1	<1	<1	5.0	6.0
10^{-6}	<1	<1	<1	6.0	16.5
10^{-5}	<1	<1	<1	10.5	18.5
10^{-4}	<1	<1	<1	4.5	4.5

only marginally inferior (16.5% benzidine positive). At an ATRA concentration of 1 x 10^{-4}M, the number of haemoglobinized cells declined to 4.5%. A remarkable finding was the range over which ATRA could induce differentiation, albeit some of it low. The data presented indicate that ATRA is effective over, at least, a 4 - log range of concentrations from 1 x 10^{-4} M to 1 x 10^{-8}M.

3.4.2.3 Sodium Butyrate (Na Bu)

A stock solution of Na Bu was prepared at 20 mM as described in section 2.7.4. REL-C7 cells were exposed to Na Bu over a range of concentrations from 0.05 mM to 5 mM. Data from 2 experiments are presented in Table 12.

Na Bu induced, at best, only a modest percentage of benzidine positive cells. The maximum obtained was 4% with Na Bu at a concentration of 1 mM. In effect, Na Bu does not appear to be a particularly useful inducer of erythroid differentiation in REL-C7 cells.

3.4.2.4 N-methylformamide (NMF) and Cytosine arabinoside (ara-C)

Two further compounds were investigated for their ability to induce REL-C7 cells to differentiate, viz, NMF (N-methylformamide) and ara-C (cytosine arabinoside).

Cells were exposed to NMF over the range 1 x 10^{-1}M to 1 x 10^{-4}M. There was no significant induction of benzidine positive cells, i.e. no NMF concentration induced >1% benzidine positive cells i.e. same as background. The cells were still functional when incubated with NMF as demonstrated by the fact that they retained high viability by trypan blue exclusion (data not shown). In addition, the cells continued to proliferate, albeit slightly suboptimally as compared to control cultures (control culture doubling time: 13.0 hr; 10^{-1}M NMF culture doubling time: 13.9 hr). Clearly, therefore, the absence of induction of differentiation was not due to some non-specific toxic effect of NMF.

Table 12

Effect of various concentrations of Na Bu on erythroid differentiation of REL-C7.

Percentage of cells staining positive for the benzidine reaction.

[NaBu] (mM)	0 hr	24 hr	48 hr	72 hr	96 hr
0	<1	<1	<1	<1	<1
0.05	<1	<1	1	2	<1
0.10	<1	<1	1	3	<1
0.50	<1	<1	1	2	<1
1.0	<1	<1	2	4	<1
5.0	<1	<1	1.5	1	<1

Initially, ara-C cultures were set up over a range of concentration from 1 x 10^{-4}M to 1 x 10^{-9}M. However, at concentrations of 1 x 10^{-4}M and 1 x 10^{-5}M., excessive cell death occurred. At concentrations of 1 x 10^{-6}M and lower, cell viability was unaffected. As with NMF, ara-C was unable to induce erythroid differentiation i.e. no ara-C concentration induced >1% benzidine positive cells. Only at ara-C 1 x10^{-6}M was proliferation slightly suboptimal.

In conclusion, DMSO was shown to be an effective inducer of erythroid differentiation in REL-C7 cells. HMBA and ATRA could also induce differentiation, though not to the same extent as DMSO. Other compounds studied were less effective. Na Bu produced only minimal levels of haemoglobinized cells, while NMF and ara-C were completely ineffective.

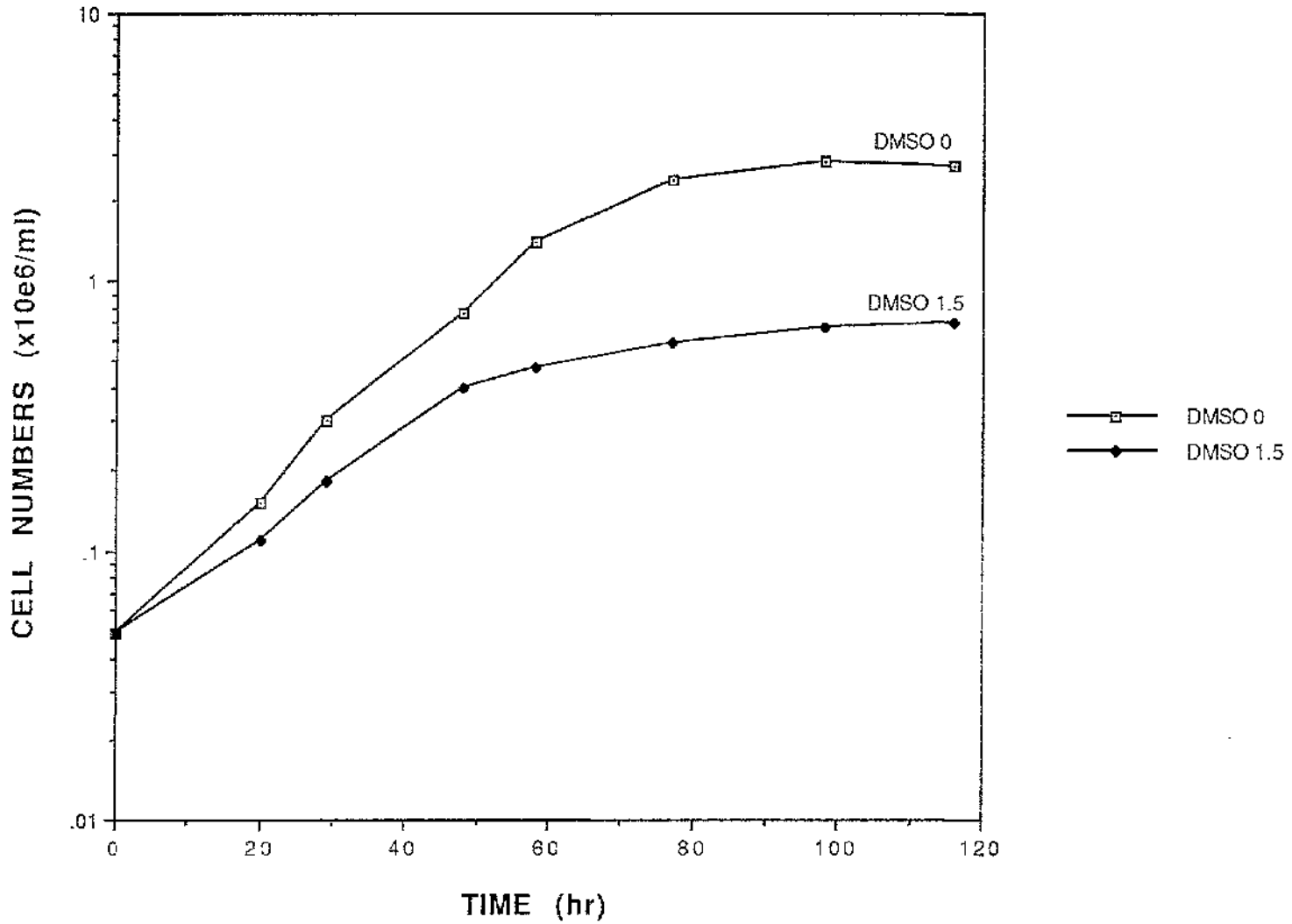
3.4.3 Effect of DMSO - induced differentiation on proliferation of REL-C7 cells

Generally speaking, leukaemic cell lines, like REL-C7, appear to have undergone some sort of maturation arrest by which the normal process of differentiation has been blocked. For example, morphologically, REL-C7 cells seem to be arrested at an early, proerythroblast(?) stage. In normal haematopoiesis there is an inter-relationship between proliferation and differentiation, in that immature, undifferentiated cells have extensive proliferative potential. As cells differentiate towards more mature forms, they progressively lose this proliferative capacity until, as end-stage cells, they are incapable of further cell division.

In the previous section, it was demonstrated that REL-C7 cells could be induced to differentiate and proceed to a more mature erythroid morphology. To assess the effects of DMSO on cell proliferation, REL-C7 cell numbers were measured over the induction period and compared to control cells. Cell counts were done using a haemocytometer (Section 2.15). A typical experiment is shown in Figure 6.

The growth curve for proliferating REL-C7 cells comprised a period of exponential growth (log phase) followed by a plateau when no further proliferation

Fig.6
Growth curves of REL-C7 cells cultured with and without 1.5% DMSO.



occurred (stationary phase). From Figure 6 it can be seen that when the cell density reached approximately 1.5 x 10^6/ml the rate of increase in cell numbers began to decrease rapidly, reaching a maximum of about 2.8 x 10^6/ml. After this point, if the cultures were not fed, the cells began to die. The doubling time in the log phase was calculated (Section 2.15). Over a 60 hr incubation period, the cell numbers doubled five times:-

Time 0	Count 0.05 x 10^6/ml	
	↓	
	0.10	Doubling # 1
	↓	
	0.20	Doubling #2
	↓	
	0.40	Doubling #3
	↓	
	0.80	Doubling #4
	↓	
Time 60	1.60 x 10^6/ml	Doubling #5

This gives an average cell doubling time of 12hr.

When cells were exposed to 1.5% DMSO, a clear difference in the growth kinetics could be seen. Again, a bi-phasic growth pattern was obtained. Over the first 48 hr of culture, the cells continued to grown exponentially, albeit with a longer doubling time than control cells grown in DMSO - free medium. In the presence of DMSO, the doubling time was extended to 16 hr. Over the next 12 hours, the rate of cell division decreased and a second phase of linear growth appeared. In this second phase, only a modest, but nevertheless measurable, increase in cell numbers was obtained. The final cell density of 0.76 x 10^6/ml was much less than control cultures. Clearly, DMSO had a profound effect on both the rate and extent of proliferation of

REL-C7 cells. It has previously been shown (Section 3.4.1) that, following exposure to DMSO, REL-C7 cells can differentiate to more mature cells, committed to the erythroid lineage. Data are presented which show that as these cells are induced to differentiate, their proliferative ability declines. This can be seen most clearly after 72hr and 96 hr of incubation in the presence of DMSO. At these time points, high levels of benzidine positive cells were present (e.g. 64% benzidine positive at DMSO 1.5% / Time 96hr). At these same time points, cell proliferation was greatly reduced. For example over a 36hr period (Time 60hr → Time 96hr) the cell numbers increased only modestly from 0.52 x 10^6/ml to 0.70 x 10^6/ml .

It is interesting to note that, although significant numbers of benzidine positive cells did not appear until 72 hr of incubation, DMSO exerted its inhibitory effects on REL-C7 proliferation from a much earlier time point. From Figure 8, the first time point of the growth curve was after 18 hr of culture. Already, proliferation in the presence of DMSO had been reduced compared to the control culture. This effect continued and became more obvious. It would appear that the production of haemoglobin is a relatively late event, and, in fact, DMSO exerts profound effects on REL-C7 cells from a very early stage.

To investigate further the mechanism of induction, REL-C7 cells were exposed to DMSO and assessed for their clonogenicity (Section 3.2) and their tumourogenicity (Section 3.3).

3.4.4 Effect of DMSO on clongenicity of REL-C7

To look at the effect of DMSO on the proliferative ability of REL cells, clonogenic assays were set up. Multiple cultures were established at a cell concentration of 5 x 10^4/ml in MEM-O++/1.5% DMSO (see section 2.7.1), total volume 5 ml. After the appropriate exposure time, the contents of one flask were transferred to a 15 ml centrifuge tube and the cells spun down (100g for 10min). The cell pellet was resuspended in a small volume (~1ml) of MEM-O++ and the final volume adjusted to 10ml, again with MEM-O++. The cells were spun down (100g for

10min). This washing procedure was repeated once more, and the final cell pellet resuspended in ~1ml MEM-O++. For the clonogenic assay, cells were routinely suspended in methyl cellulose/FBS at a concentration of 1 x 10^3/ml. Aliquots of 1 ml were dispensed into 35 mm suspension culture dishes and incubated under the usual conditions. After a 96 hr incubation, the plates were scored for the number of colonies (colony >50 cells). The data from 5 experiments are summarised in Table 13.

Clearly, DMSO has a profound effect on the clongenicity of REL-C7 cells. After as little as 2 hr exposure to DMSO, a small but detectable, decrease in the cloning efficiency was observed (49.5% clonogenicity cf. 54% in controls). The DMSO-4hr sample showed a further reduction in cloning efficiency (down to 47.2%). After 6hr exposure to DMSO, a further, this time significant, reduction in cloning efficiency was measured (Student's t-test; $p<0.05$). Figure 7 shows that this trend continued with a linear reduction in cloning efficiency up until 24 hr of DMSO exposure (clonogenicity 5%). The DMSO-48 hr sample showed only minimal cloning efficiency, 0.325%.

These results demonstrate that as REL-C7 cells were increasingly induced to differentiate, proliferation was concomitantly down - regulated. Analysis of single cells by the clonogenic assay revealed that 24 hr exposure to DMSO prevented 95% of REL cells from proliferating normally, and by 48 hr exposure >99% were non-proliferative. From Figure 7, the linear reduction in cloning efficiency indicates that DMSO exerts its influence on REL cells immediately (i.e. no detectable lag time). Indeed, a statistically significant reduction in cloning efficiency was observed after only 6hr exposure to DMSO. Thus, although DMSO-committed REL cells may require some days to accumulate detectable haemoglobin, early events take place within the first few hours that have a profound effect on proliferation and the expression of the differentiated phenotype.

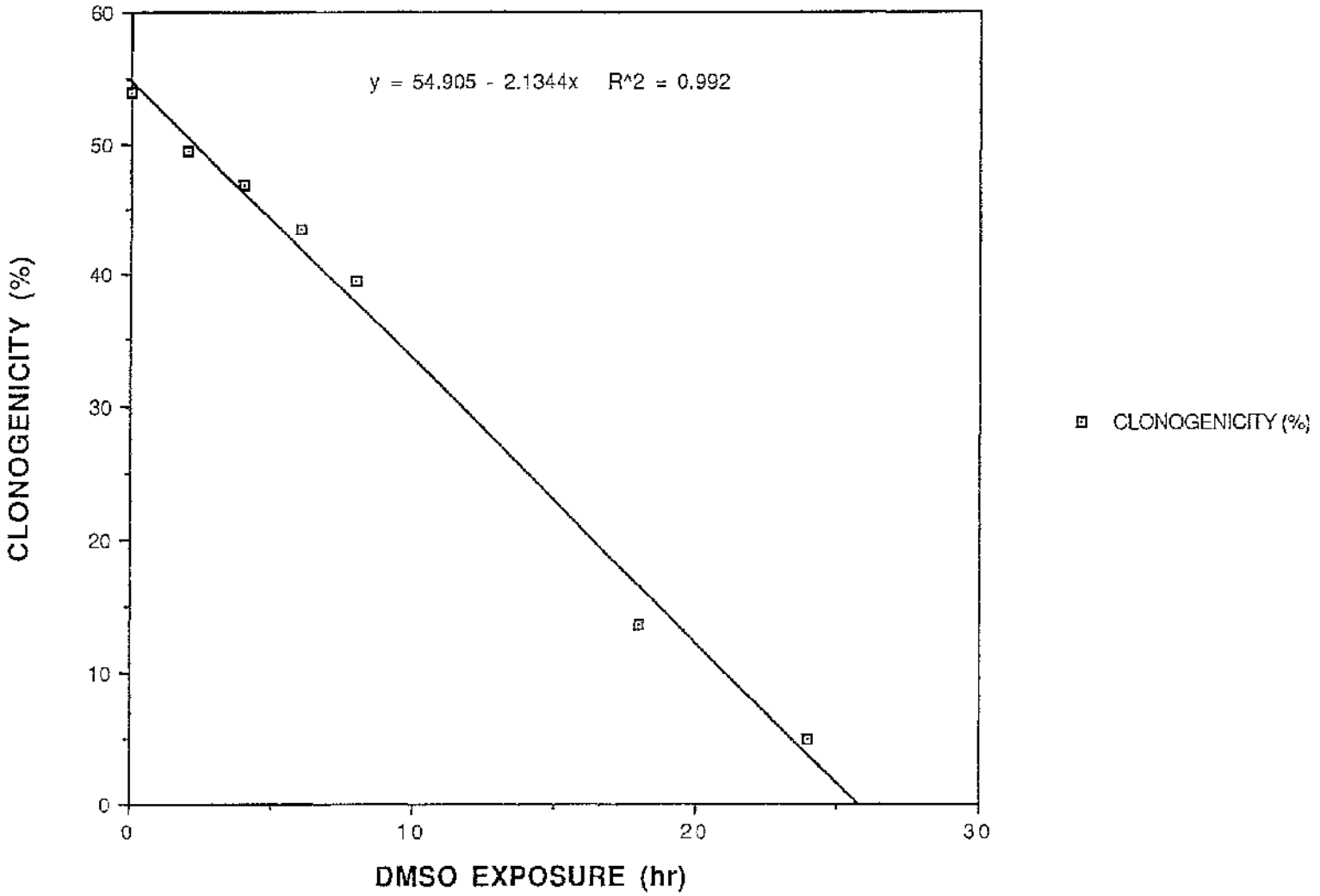
Table 13

Effect of various exposure times of DMSO on REL-C7 clonogenicity.

Data are expressed as the mean ± SEM of five experiments.

Exposure Time to DMSO (hr)	% Clonogenicity
0	54 ± 3.34
2.	49.5 ± 2.40
4	47.2 ± 2.06
6	43.3 ± 3.20
8	39.5 ± 0.50
18	13.3 ± 2.67
24	5.0 ± 1.15
48	0.325 ± 0.125

Fig.7
REL-C7 cells exposed to 1.5% DMSO for various periods of time. Effect on clonogenicity.



3.4.5 Effect of DMSO on tumourogenicity of REL-C7

One of the key properties of REL-C7 cells is their malignancy. As previously shown (Section 3.3) REL cells can readily proliferate *in vivo*, producing tumours when injected into Long Evans babes.

The preceding experiments have demonstrated that, in *in vitro* assays, REL-C7 cell proliferation can, to all intents and purposes, be switched off. Experiments were set up to investigate if REL-C7 cells exposed to DMSO similarly lost their malignant potential. i.e. their ability to produce tumours.

In the first series of experiments, REL-C7 cells were exposed to 1.5 % DMSO for 96hr to achieve maximum levels of differentiated, haemoglobinized cells. At the end of this incubation period, cells were washed twice as before and finally resuspended in a small volume (~1ml) of MEM-O++. Appropriate cell dilutions were made to enable a range of cell doses to be administered to recipient babes (see Section 2.6). Prior to injection, an aliquot of these cells was always stained with the benzidine reagent to ensure that they had indeed differentiated. Invariably, between 60% and 70% of the cells stained benzidine positive. At the same time, control babes received a range of REL-C7 cells that had not been exposed to DMSO. Recipient animals were monitored regularly for the appearance of tumour (Table 14).

Overall, there was a striking reduction in the tumourogenic potential of REL-C7 cells. A group of 13 animals each received 1 x 10^4 control REL cells. In accordance with previous experiments (Section 3.3), 100% of animals developed tumour within 17 days of injection (cf. previous data: 100% tumour incidence by day 15). In contrast, of the animals which received 1 x 10^4 DMSO- treated cells, only 43% (3/7) developed tumour. Furthermore, in those animals, there was a profound delay before tumour first appeared. In the control group, tumour was first detected on d.7 and all animals had succumbed by d17. In the DMSO group, the first tumour did not appear until d.19 and no further animals developed tumour after d.29.

A group of 12 animals each received 1 x 10^3 control REL cells. The results were in general agreement with previous studies. As before, there was 100%

Table 14

Incidence of tumour formation following administration of DMSO-treated REL-C7.

Days post-injection of REL-C7

Cells injected per babe	0	7	9	11	13	15	17	19	21	23	25	27	29	%Overall Incidence
10^4 Control	0 / 13	2 / 13	3 / 13	6 / 13	11 / 13	12 / 13	13 / 13							100%
10^4 DMSO	0 / 7	0 / 7	0 / 7	0 / 7	0 / 7	0 / 7	0 / 7	1 / 7	1 / 7	2 / 7	2 / 7	2 / 7	3 / 7	43%
10^3 Control	0 / 12	0 / 12	3 / 12	8 / 12	9 / 12	11 / 12	12 / 12							100%
10^3 DMSO	0 / 8												0 / 8	0%
10^2 Control	0 / 15	0 / 15	0 / 15	1 / 15	4 / 15	9 / 15	11 / 15	12 / 15	12 / 15	14 / 15	14 / 15	14 / 15	14 / 15	93%
10^2 DMSO	0 / 8												0 / 8	0%
10^1 Control	0 / 17	0 / 17	0 / 17	0 / 17	0 / 17	1 / 17	4 / 17	5 / 17	8 / 17	8 / 17	9 / 17	9 / 17	9 / 17	53%
10^1 DMSO	0 / 9												0 / 9	0%

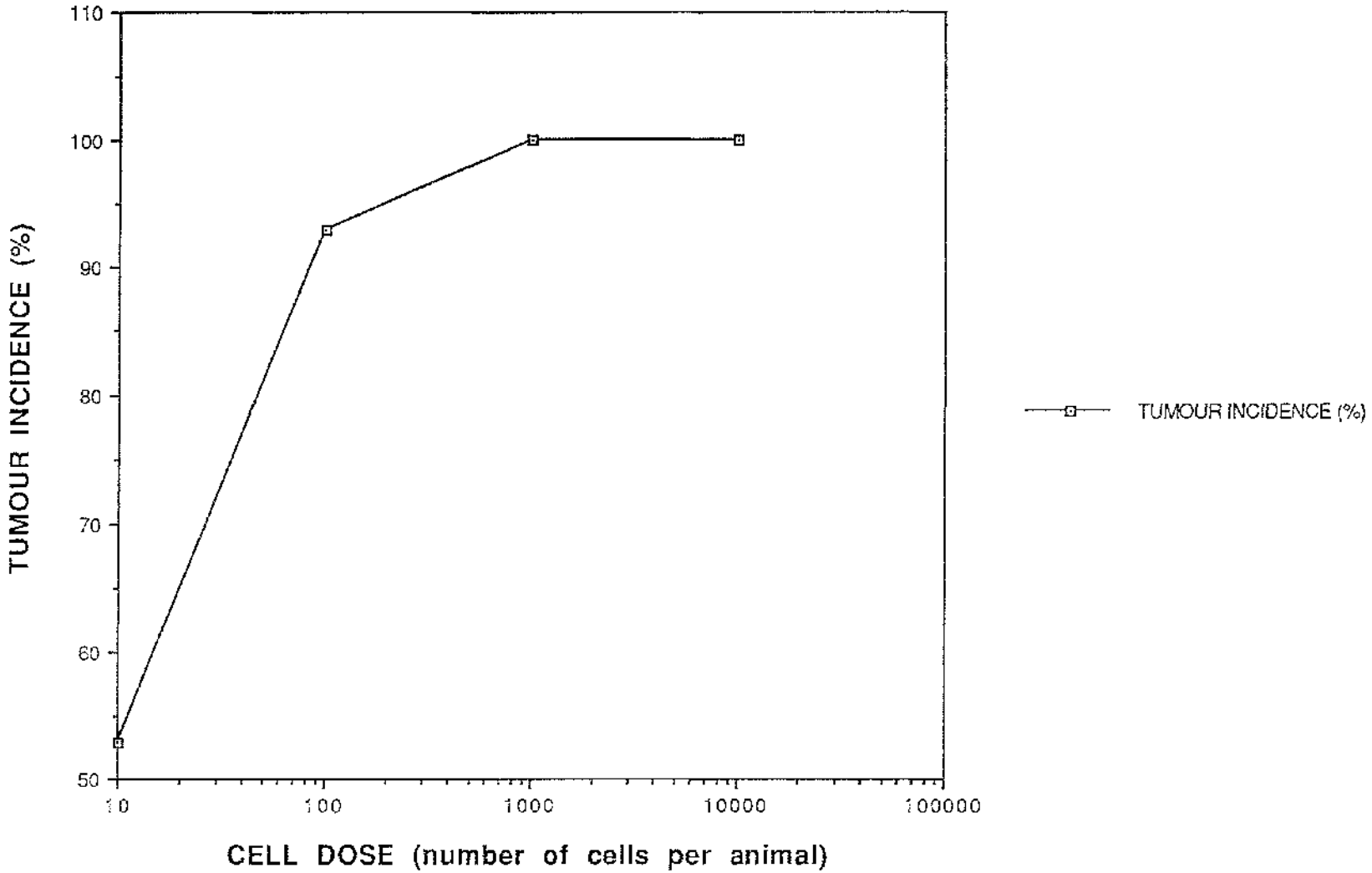
incidence of tumour formation. Tumours first appeared on d.9 (cf d.13 in Section 3.3), and by d.17 all animals had succumbed (cf. d.23 in Section 3.3). In contrast, in the DMSO group of 8 animals, no tumours developed.

A group of 15 animals each received 1 x 10^2 control REL cells. The incidence of tumour was 93% (14/15) (cf. 96% (23/24) in Section 3.3). The first tumour appeared on d11 (cf. d 15 in Section 3.3) and tumours continued to develop until d.23 (cf. d.25 in Section 3.3). Again, in the DMSO group, no tumour was detected in any of 8 animals.

The last group comprised 17 animals and each received 1 x 10^1 control REL cells. The tumour incidence was 53% (9/17) (cf. 45% (10/22) in Section 3.3).The first tumour appeared on d.15 (cf. d.19 in Section 3.3) and no further tumour was detected beyond d.25 (cf. d.25 in Section 3.3). There was no incidence of tumour in any of the 9 animals that received 1 x 10^1 DMSO-treated cells.

From a validation point of view, all the control groups corroborated the results of the larger study in Section 3.3, both in the time course of tumour detection and in the overall incidence of tumour in the various groups. Across the groups of animals that received DMSO-treated cells, there was a remarkable reduction in tumourogenicity. The number of animals in each group was small, making accurate analysis difficult. However, some general observations can be made. In Section 3.3 the TD_{50} was calculated to be 12 cells/babe. In the present series of experiments the TD_{50} was 9 cells (Figure 8). Indeed, if the two sets of data are combined, a TD_{50} of 10 cells is obtained. Using DMSO-treated cells, it was only in the group of animals that each received 1 x 10^4 cells that any tumour developed (45% incidence). Control experiments demonstrated that, in animals receiving 10 undifferentiated REL-C7 cells, a tumour incidence of 50% could be expected. In those animals that received 1 x 10^4 DMSO-treated REL-C7 cells, the incidence of tumour was not dissimilar to those control animals i.e. 43% cf 50%. These data indicate that only a small proportion of the DMSO-treated REL-C7's have retained their tumourogenic potential. To extrapolate from the control experiments, an inoculum of 1 x 10^4

Fig.8
TD50 for L.E. babes injected with various concentrations of DMSO-treated REL-C7 cells.



DMSO-treated cells may contain approximately 10 cells which still retain sufficient proliferative potential to give rise to tumour, i.e. only 1 in 1,000. This represents a remarkable reduction in the number of clonogenic malignant cells.

Clearly, DMSO had a profound effect on the proliferation of REL-C7 cells. Cell proliferation was rapidly and effectively down-regulated as demonstrated by *in vitro* cell culture. In particular, single cell analyses (clonogenic assay) indicated that when REL-C7 cells were exposed to DMSO for 48 h, only 0.325% (i.e. 3 in 1,000) retained any proliferative ability. Similarly, DMSO exposure markedly reduced the ability of REL-C7 cells to form tumours in recipient animals.

Taken together, these data indicate that as DMSO induces REL-C7 cells to differentiate, there is a reciprocal loss of proliferative potential. After 4 days of culture, the majority of cells are phenotypically mature erythroid with few retaining any proliferative ability.

3.5 Commitment to erythroid differentiation

Data have been presented demonstrating that DMSO can induce REL-C7 cells to differentiate and synthesize haemoglobin in culture. On the whole, the end-point of these experiments was to assess the cells for haemoglobin production after 4 days of exposure to DMSO. However, it was clear that, although some 96 hours may be required for maximum production of haemoglobin, a significant down-regulation in cell division and proliferation could be detected after only a few hours exposure to DMSO. Experiments were set up to investigate this induction process by analysing the kinetics of commitment to differentiation.

Commitment is defined as the ability to continue differentiation in the absence of inducing agent.

REL-C7 cells were exposed to DMSO for various defined periods of time, then the DMSO was removed and the cells were monitored to see if they continued to differentiate or if they reverted to the undifferentiated phenotype. The fate of these cells was followed by several different assessments: -

1. Single cell analysis,

2. ^{3}H-thymidine incorporation,

3. Cell cycle analysis,

4. Cell size,

5. Scanning electron microscopy

3.5.1 Single Cell Analyses

To investigate the kinetics of commitment to differentiation, single cell analyses were performed. These experiments were based on the limiting dilution studies set up for cloning REL cells (Section 2.3.1).

Experiments were set up to establish the most convenient cell concentration to use. With the cell concentration at, nominally, 10 cells/ml, aliquots of 100 µl were dispensed into the wells of a 96-well tissue culture plate, i.e. "1 cell/well". Data from 2 experiments are shown in Figure 9.

A total of 120 wells each received 100 µl of REL-C7 at 10 cells/ml, i.e. a total of 120 cells were dispensed. After 4 days of culture, the wells were examined for colony formation. Any cells which had proliferated to form a clone were easily detected. Furthermore, some wells, which were positive for growth, contained more than one clone. These multiple clones/well could be readily identified, thus minimising any counting inaccuracy.

From Figure 9, it can be seen that from, theoretically, 120 cells, 63 clones were obtained, i.e. 52.5% clonogenicity. This value is remarkably close to the previous cloning efficiency value of 55-59% (Section 3.2). Fifty-nine per cent of the wells (71 out of 120) contained no clones. Thirty-two percent of the wells (38 out of 120) contained a single clone and nine percent of the wells (11 out of 120) contained multiple clones (8 wells contained 2 clones; 3 wells contained 3 clones). The incidence of multiple clone wells (9%) was deemed to be unacceptably high and so further experiments were set up at a reduced concentration of 0.5 cell/well. Data from 4 experiments are shown in Figure 10.

Figure 9

Limiting dilution cultures of REL-C7 cells with 1 cell / well.

Number of clones per well.

Plate 1

0	0	0	0	0	0	0	0	0	0
1	1	0	2	0	1	0	1	0	3
1	0	0	1	1	1	0	0	0	1
0	0	3	0	0	2	0	1	1	1
1	1	0	1	2	1	0	1	0	0
0	0	0	1	1	0	0	1	0	1

Plate 2

0	0	1	2	2	3	0	0	0	0
1	0	1	2	0	0	0	0	0	1
1	0	0	0	0	0	0	0	0	1
0	0	0	0	2	0	1	0	1	0
0	0	0	1	1	2	1	0	1	1
0	0	1	1	0	0	1	0	1	0

Range (clones per well) : 0 - 3.

Total number of clones : 63

Total number of cells plated : 120

Overall clonogenicity : 52.5% (63/120)

Frequency : 0 clones per well : 71 (59% of wells)

: 1 clone per well : 38 (32% of wells)

: 2 clones per well : 8 (6.7% of wells)

: 3 clones per wel : 3 (2.5% of wells)

Figure 10

Limiting dilution culture of REL-C7 cells with cells at 0.5 cell / well.

Number of clones per well.

Plate 1

0	1	0	0	0	0	0	0	0	0
0	0	0	1	0	0	0	0	1	0
0	1	0	0	1	0	1	0	0	0
0	0	0	0	0	1	1	0	0	0
0	0	1	0	0	0	0	0	0	0
1	0	1	1	0	0	0	0	0	2

Plate 2

0	0	0	1	1	1	1	0	1	0
1	1	0	1	1	0	0	1	0	1
0	0	0	0	1	0	0	0	0	0
1	1	0	0	0	0	0	0	0	0
1	0	1	0	0	0	0	0	1	1
1	0	0	2	0	0	1	0	0	0

Plate 3

0	1	0	0	0	0	0	1	1	0
1	2	0	2	1	1	0	0	1	1
0	1	1	0	0	0	0	0	0	0
0	1	0	0	0	0	1	0	0	0
1	0	0	0	1	0	0	0	0	0
0	1	0	0	1	1	0	1	0	0

Plate 4

0	0	0	0	0	1	0	1	0	1
0	1	0	0	0	0	1	0	0	0
1	0	0	0	0	0	0	1	1	0
0	0	0	2	2	0	0	0	0	0
1	0	0	0	0	0	0	1	0	1
0	0	0	0	1	0	0	0	0	0

Range (clones per well) : 0 - 2

Total number of clones : 74

Total number of cells plated : 120

Overall clonogenicity : 61.7% (74/120)

Total number of wells : 240

Frequency : 0 clones per well : 172 (72% of wells)

: 1 clone per well : 62 (26% of wells)

: 2 clones per well : 6 (2.5% of wells)

The overall cloning efficiency was 61.7% (75 clones from 120 cells). Again, this compared most favourably with the previously established value of 55-59% (Section 3.2). Using this reduced cell concentration, 72% of the wells (172 out of 240) contained no clones; 26% of the wells (62 out of 240) contained a single clone; only 2.5% of the wells (6 out of 240) contained 2 clones. No wells contained greater than 2 clones. Due to the low incidence of multiple clone wells, a cell plating concentration of 0.5 cell/well was chosen as the most convenient for subsequent analyses.

3.5.2 Spontaneous erythroid differentiation

As previously reported (Section 3.4.1), REL-C7 cells demonstrate a low background level of spontaneous differentiation i.e. differentiation in the absence of any inducer. In DMSO-free culture, the number of benzidine positive cells rarely exceeded 1%.

In an effort to investigate the origin of these differentiating cells, limiting dilution cultures were set up at 0.5 cell/well. Multiple 96-well plates were set up and the contents of the wells were analysed after 24 hr, 48hr, 72hr, 96hr, 120 hr and 144 hr of incubation. At the appropriate time point, individual well cultures were terminated by the addition of the benzidine reagent (see Section 2.9). Providing that the benzidine was added slowly and carefully, the cells in the well were not significantly disturbed. For each well, the total number of cells was counted and the number of benzidine positive cells was counted. Data are presented in Tables 15A-15F, and summarised in an additional table.

After 24hr, 48hr, 72hr and 96 hr of culture (Tables 15A, B, C, D) it was possible to count the number of cells in each individual colony. Because the cells were cultured in liquid medium, colonies tended to be disperse in appearance as opposed to the 3-dimensional ball of cells seen when cells were cultured in methyl cellulose. This dispersed morphology facilitated counting. Obviously with the bigger colonies obtained after 120 hr and 144 hr of culture, counting of numbers of cells per

Table 15

Benzidine staining of individual REL-C7 clones cultured in the absence of inducer.

Assessment at various time points.

15 A. 24 hr of culture

Clone #	Total no. of cells	No. of B+ve cells
1	4	0
2	4	0
3	2	0
4	8	0
5	4	0
6	4	0
7	4	0
8	3	0
9	4	0
10	4	0
Total no. of cells	**41**	**0**

∴ Overall incidence of B+ve cells : 0%

Incidence of colonies containing B+ve cells : 0%

Mean no.of cells per colony (mean ± SD) : 4.1 ± 1.5

Median " " " : 4

Clonogenicity (60 wells ≡ 30 cells) : 33% (10 / 30)

Table 15 B. **48 hr of culture**

Clone #	Total no. of cells	No. of B+ve cells
1	4	0
2	8	0
3	6	0
4	9	0
5	20	0
6	4	0
7	6	0
8	10	0
9	12	0
10	16	0
11	6	0
12	8	0
13	9	0
14	12	0
15	8	0
16	8	0
Total no. of cells	**146**	**0**

∴ Overall incidence of B+ ve cells : 0%

Incidence of colonies containing B+ve cells : 0%

Mean no.of cells per colony (mean ± SD) : 9.1 ± 4.2

Median " " " : 8

Clonogenicity (60 wells ≡ 30 cells) : 53% (16 / 30)

Table 15C. **72 hr of culture**

Clone #	Total no. of cells	No. of B+ve cells
1	32	0
2	30	0
3	34	0
4	22	0
5	60	4 (≡ 6.7%)
6	22	0
7	8	0
8	48	0
9	30	0
10	26	0
11	60	0
12	36	0
13	38	3 (≡8%)
14	28	0
Total no. of cells	**474**	**7**

∴ Overall incidence of B+ve cells : 1.5% (7 / 474)

Incidence of colonies containing B+ve cells : 14% (2 / 14)

Mean no.of cells per colony (mean ± SD) : 34 ± 14

Median " " " : 31

Clonogenicity (60 wells ≡ 30 cells) : 47% (14 / 30)

Table 15 D. **96 hr of culture**

Clone #	Total no. of cells	No. of B+ve cells
1	60	0
2	25	0
3	~200	0
4	120	0
5	30	0
6	150	8 (≡5%)
7	80	0
8	~200	0
9	~200	0
10	100	0
11	50	0
12	60	0
13	50	6 (≡12%)
14	150	0
15	40	0
16	40	0
17	~260	10 (≡3.8%)
18	80	0
19	100	0
20	150	0
21	46	0
22	36	0
23	~200	6 (≡3%)
24	60	0
Total no. of cells	**~2,500**	**30**

∴ Overall incidence of B+ve cells	:	1.2% (30 / 2,500)
Incidence of colonies containing B+ve cells	:	17% (4 / 24)
Mean no.of cells per colony (mean ± SD)	:	104 ± 69
Median " " "	:	80
Clonogenicity (90 wells ≡ 45 cells)	:	53% (24 / 45)

Table 15 E. **120 hr of culture**

Clone #	Total no. of cells	No. of B+ve cells
1	~200	0
2	~200	0
3	~200	0
4	~200	0
5	~250	0
6	~500	0
7	25	0
8	~1,000	0
9	~300	30 (≡10%)
10	~1,000	0
11	150	0
12	150	0
13	150	8 (≡5%)
14	~200	0
15	100	5 (≡5%)
16	50	25 (≡50%)
17	~1,000	0
18	~200	0
19	~1,000	0
20	~500	0
21	150	0
22	~1,000	25 (≡2.5%)
Total no. of cells	**~10,000**	**93**

∴ Overall incidence of B-I-ve cells	:	0.93% (93 / 10,000)
Incidence of colonies containing B-I-ve cells	:	23% (5 / 22)
Mean no.of cells per colony (mean ± SD)	:	454 ± 356
Median " " "	:	225
Clonogenicity (90 wells ≡ 45 cells)	:	49% (22 / 45)

Table 15 F. **144 hr of culture**

Clone #	Total no. of cells	No. of B+ve cells
1	~1,000	0
2	~200	0
3	~2,000	0
4	~200	14 (≡7%)
5	~2,000	36 (≡1.8%)
6	60	0
7	~1,000	20 (≡2%)
8	~2,000	8 (≡0.4%)
9	~3,000	0
10	~1,000	0
11	60	56 (≡90%)
12	~2,000	0
13	~1,000	0
14	~2,000	10 (≡0.5%)
15	~1,000	0
16	~1,500	0
17	~2,000	53 (≡2.7%)
18	~200	10 (≡5%)
Total no. of cells	**~22,000**	**207**

∴ Overall incidence of B+ve cells : 0.94% (207 / 22,000)

Incidence of colonies containing B+ve cells : 49% (8 / 18)

Mean no.of cells per colony (mean ± SD) : 1222 ± 873

Median " " " : 1,000

Clonogenicity (60 wells ≡ 30 cells) : 60% (18 / 30)

Table 15 Summary

Duration of Culture(hr)	Total no. of cells	No. of B+ve cells	% B+ve	Incidence of cols containing B+ve cells (%)	Median no. of cells per colony	Cloning efficiency (%)
24	41	0	0	0	4	33
48	146	0	0	0	9	53
72	474	7	1.5	14	31	47
96	~2,500	30	1.2	17	80	53
120	~10,000	93	0.93	23	225	49
144	~20,000	207	0.94	44	1,000	60

colony became less accurate. As an aid to counting, similar sized colonies were often pooled, spun down to a smaller volume and a manual cell count (haemocytometer) performed. The total number of cells was then divided by the number of input colonies to give an average number of cells per colony. This proved useful in estimating the approximate number of cells in colonies > 250 cells.

From Tables 15A-F, it could be seen that the overall cloning efficiency was similar to previous experiments: - 24 hr culture, 33% (10/30); 48 hr culture, 64% (16/30); 72 hr culture, 57% (14/30); 96 hr culture, 53% (24/45); 120 hr culture, 49% (22/45); 144 hr culture, 60% (18/30).

Likewise, cell proliferation, as assessed by the doubling times, was similar to earlier experiments. For each of the six time-points, the mean number of cells per colony was calculated, e.g. for the 72 hr culture, a total of 474 cells were counted from 14 colonies giving a mean of 34 cells per colony (474/14). Between the 24 hr culture and the 48 hr culture, the doubling time was 21 hr. Between the 48 hr culture and the 72hr culture, the doubling time was 12.7 hr. Between the 72 hr culture and the 96 hr culture, the doubling time was 15.7 hr. Between the 96 hr culture and the 120 hr culture, the doubling time was 11.5 hr. Between the 120 hr culture and the 144 hr culture, the doubling time was 17.8 hr.

When compared to the previously established doubling time of 12-14 hr, the doubling time of 21 hr between the first two experiments is appreciably longer. This may be due to some sort of lag phase as the cells adapt to fresh culture conditions. In addition, the accuracy of this figure will be compromised by the relatively few cells that were analysed. The doubling times of 12.7 hr, 15.7 hr and 11.5 hr are all similar to the established value of 12-14 hr. The value 15.7 hr is slightly high, while the value 11.5 hr is slightly low. This may reflect the inherent counting inaccuracy and the fact that relatively few cells were analysed. The final doubling time of 17.8 hr was significantly longer than the normal 12-14 hr. In addition to the inaccuracies outlined above, it is likely that necessary nutrients in the culture medium were

becoming increasingly depleted after 6 days of culture. This would account for the rate of proliferation slowing down.

The overall incidence of benzidine positive (B+ve) cells was calculated by counting the number of B+ve cells within colonies and dividing this figure by the total number of cells in all the wells of each experiment. As expected, a small but detectable proportion of cells gave rise to some B+ve cells. The overall incidences of B+ve cells were:-

24 hr culture	:	0%	(0/41)
48 hr culture	:	0%	(0/146)
72 hr culture	:	1.5%	(7/474)
96 hr culture	:	1.2%	(30/2,500)
120 hr culture	:	0.93%	(93/10,000)
144 hr culture	:	0.94%	(207/22,000)

Interestingly, no B+ve cells were detected in either the 24 hr or 48 hr culture. As discussed earlier, this may reflect the low numbers of cells analysed. The first B+ve cells were detected in the 72 hr culture and the numbers continued to rise in the subsequent culture. The proportion of B+ve cells declined over time. This is to be expected since any cells that differentiate will lose their proliferative ability, unlike the majority of cells which do not differentiate and continue to divide, thus increasing the proportion of B-ve cells.When the incidence of colonies containing B+ve cells was examined, a pattern emerged, whereby as the culture period increased then, the proportion of colonies containing B+ve cells correspondingly increased:-

24 hr culture	:	0%	(0/10)
48 hr culture	:	0%	(0/16)
72 hr culture	:	14%	(2/14)
96 hr culture	:	17%	(4/24)
120 hr culture	:	23%	(5/22)
144 hr culture	:	44%	(8/18)

If, as it seems, this spontaneous differentiation is a chance event, then this trend is to be expected. The longer the cells are cultured, the more cells will be produced, increasing the probability that a cell will, by chance, spontaneously differentiate.

In those colonies that did contain B+ve cells, the actual proportion of B+ve cells ranged from <1% of the total cells in a colony up to ~ 90% of the cells. The reciprocal relationship between proliferation and differentiation can be amply demonstrated if all the colonies that contained B+ve cells are arranged in order of % B+ve (Table 16). The general trend is that as the proportion of B+ve cells per colony increases then the total number of cells per colony correspondingly decreases. Thus in any given colony, an increase in the number of differentiating cells is accompanied by a decrease in the number of proliferating cells.

Thus, spontaneous differentiation occurs in a minority of cells and appears to be a chance event.

3.5.3 DMSO - induced erythroid differentiation

In the previous section, the spontaneous differentiation of REL-C7 cells was investigated by examining single cells cultured in the absence of inducer in 96-well tissue culture plates. As a follow-on from this, similar experiments were set up whereby REL-C7 cells were exposed to DMSO (1.5%) for defined periods of time before being plated out at limiting dilution (see Section 2.9).

Multiple cultures of REL-C7 cells in DMSO-containing medium were established. Cells were exposed to DMSO for the following times:- 4 hr, 8 hr, 16 hr, 24 hr, 32 hr and 48 hr. After the appropriate period of time had elapsed, DMSO exposure was terminated by washing the cells free from DMSO and resuspending them in MEM-O ++ alone (i.e. no DMSO). The cell concentration was adjusted to "5 cells/ml" and aliquots of 100 µl were dispensed into the wells of a 96-well tissue culture plate (i.e. 0.5 cell/well). These cultures were maintained for 5 days, when the

Table 16

Relationship between the proportion of B+ve cells per colony and the total number of cells per colony.

% B+ve cells per colony	Total no. of cells per colony
0.4	~2,000
0.5	~2,000
2	~2,000
2	~1,000
2.5	~1,000
3	~2,000
3	~200
3.3	~260
5	~200
5	150
5	100
5	150
6.7	60
7	~200
10	~300
12	50
50	50
90	60

wells were examined for cell growth. The number of cells per well was counted /estimated and the benzidine reagent was added to detect those cells that had differentiated (Tables 17A-G, summary).

Generally speaking, the overall cloning efficiency was similar to previous experiments:- 0 hr exposure, 47%; 4 hr exposure, 60%; 8 hr exposure, 60%; 16 hr exposure, 63%; 24 hr exposure, 60 %; 32 hr exposure, 77%; 48 hr exposure 30%. Again, there will be some inaccuracy in these figures, reflecting the relatively small numbers of cells analysed. In the plate with cells exposed to DMSO for 48 hr, there may be an additional inaccuracy. From Table 17G it can be seen that there was very little cell proliferation and that in these wells identified as positive for growth, 6/9 contained only 2 cells. Clearly, when so few cells are present, it becomes difficult to detect them. Although great care was taken to scan the entire contents of each well, it is conceivable that the occasional single cell or cell doublet may have evaded detection.

As an indicator of proliferation, the median number of cells per colony was calculated for each exposure time point (see Table 17 Summary). A clear pattern emerged whereby the number of cells per colony declined with increasing exposure to DMSO, i.e. DMSO exposure down-regulated proliferation. There was no significant difference between the control cells (no exposure to DMSO) and the cells exposed to DMSO for 4 hr. However, a significant reduction in proliferation was seen in the cells exposed to DMSO for 8 hr (625 cells/colony cf~1,000 cells per colony in control cells) ($P<0.02$, Students' t-test). This reduction in number of cells per colony continued with increasing DMSO exposure times:- 16 hr exposure, 156 cells/colony; 24 hr exposure, 50 cells/colony; 32 hr exposure, 4 cells colony; 48 hr exposure, 2 cells/colony. The proliferative down- regulation appeared to be particularly profound between 24 and 32 hr of DMSO exposure (i.e. from 50 cells/colony $\rightarrow$ 4 cells/colony). In the 32 hr and 48 hr exposure experiments, the median number of cells/colony was low (4 and 2 respectively). Nevertheless, for the purposes of these analyses, they were regarded as clones.

Table 17

Benzidine staining of individual clones from cells exposed to DMSO for various periods of time then cultured in DMSO-free medium.

17A. 0 hr exposure

Clone #	Total no. of cells	No. of B+ve cells
1	~1,000	0
2	~1,000	0
3	~1,000	0
4	~1,000	43 (≡4.3%)
5	~500	16 (≡3.2%)
6	~1,000	0
7	~1,000	0
8	~1,000	0
9	~1,000	0
10	~1,000	0
11	~1,000	51 (≡5.1%)
12	~1,000	0
13	150	0
14	~1,000	0
Total no. of cells	**~12,650**	**110**

∴ Incidence of B+ve cells	:	0.87% (110 / 12,650)
Incidence of colonies containing B+ve cells	:	21% (3 / 14)
Mean no.of cells per colony (mean ± SD)	:	903 ± 255
Median " " "	:	~1,000
Clonogenicity (60 wells ≡ 30 cells)	:	47% (14 / 30)

Table 17B. **4 hr exposure**

Clone #	Total no.of cells	No. of B+ve cells
1	~1,000	0
2	~1,000	0
3	~1,000	0
4	~1,000	0
5	~1,000	0
6	~1,000	0
7	~1,000	8 (≡0.8%)
8	~1,000	0
9	~200	77 (≡39%)
10	~1,000	0
11	~1,000	0
12	~1,000	0
13	~1,000	0
14	50	6 (≡12%)
15	~1,000	0
16	~1,000	0
17	~200	23 (≡11.5%)
18	~1,000	0
Total no. of cells	**~16,000**	**114**

∴ Overall incidence of B+ve cells : 0.71% (114 / 16,000)

Incidence of colonies containing B+ve cells : 22% (4 / 18)

Mean no.of cells per colony (mean ± SD) : 900 ± 330

Median " " " : ~1,000

Clonogenicity (60 wells ≡ 30 cells) : 60% (18 / 30)

Table 17C. **8 hr exposure**

Clone #	Total no. of cells	No. of B+ve cells
1	~200	0
2	~200	0
3	~200	0
4	~200	8 (≡4%)
5	~1,000	0
6	150	27 (≡18%)
7	~1,000	0
8	112	27 (≡24%)
9	~250	18 (≡7.2%)
10	~1,000	51 (≡5.1%)
11	~1,000	24 (≡2.4%)
12	56	15 (≡27%)
13	~1,000	26 (≡2.6%)
14	~1,000	0
15	32	0
16	~1,000	9 (≡0.9%)
17	~1,000	5 (≡0.5%)
18	~1,000	0
Total no. of cells	**~10,000**	**210**

∴ Overall incidence of B+ve cells : 2.1% (210 / 10,000)

Incidence of colonies containing B+ve cells : 56% (10 / 18)

Mean no.of cells per colony (mean ± SD) : 555 ± 438

Median " " " : 625

Clonogenicity (60 wells ≡ 30 cells) : 60% (18 / 30)

Table 17D. **16 hr exposure**

Clone #	Total no. of cells	No. of B+ve cells
1	~300	20 (≡7%)
2	62	6 (≡10%)
3	~500	0
4	~200	0
5	~500	12 (≡2.4%)
6	~200	11 (≡5.5%)
7	64	0
8	~400	0
9	117	0
10	16	16 (≡100%)
11	10	10 (≡100%)
12	~1,000	30 (≡3%)
13	~500	21 (≡4%)
14	10	5 (≡50%)
15	40	0
16	156	37 (≡24%)
17	~300	16 (≡5.3%)
18	142	6 (≡4.2%)
19	34	7 (≡21%)
Total no. of cells	**~4,500**	**197**

∴ Overall incidence of B+ve cells : 4.4% (197 / 4,500)

Incidence of colonies containing B+ve cells : 68% (13 / 19)

Mean no.of cells per colony (mean ± SD) : 240 ± 253

Median " " " : 156

Clonogenicity (60 wells ≡ 30 cells) : 63% (19 / 30)

Table 17E. **24 hr exposure**

Clone #	Total no. of cells	No. of B+ve cells
1	~500	0
2	8	8 (≡100%)
3	~600	19 (≡3%)
4	104	53 (≡51%)
5	10	8 (≡80%)
6	42	17 (≡40%)
7	3	3 (≡100%)
8	154	0
9	10	8 (≡80%)
10	144	0
11	50	6 (≡12%)
12	~200	21 (≡10.5%)
13	~1,000	10 (≡1%)
14	5	5 (≡100%)
15	150	0
16	8	8 (≡100%)
17	3	3 (≡100%)
18	3	3 (≡100%)
Total no. of cells	**~3,000**	**172**

∴ Overall incidence of B+ve cells : 5.7% (172 / 3,000)

Incidence of colonies containing B+ve cells : 78% (14 / 18)

Mean no.of cells per colony (mean ± SD) : 170 ± 269

Median " " " : 50

Clonogenicity (60 wells ≡ 30 cells) : 60% (18 / 30)

Table 17F. **32 hr exposure**

Clone #	Total no. of cells	No. of B+ve cells
1	30	1 (=3%)
2	2	2 (=100%)
3	6	6 (=100%)
4	3	1 (=33%)
5	2	2 (=100%)
6	1	1 (=100%)
7	3	3 (=100%)
8	2	2 (=100%)
9	8	8 (=100%)
10	4	4 (=100%)
11	5	5 (=100%)
12	63	0
13	2	1 (=50%)
14	14	14 (=100%)
15	4	4 (=100%)
16	~300	62 (=21%)
17	2	2 (=100%)
18	18	14 (=78%)
19	3	3 (=100%)
20	4	4 (=100%)
21	2	2 (=100%)
22	8	8 (=100%)
23	2	2 (=100%)
Total no. of cells	**488**	**151**

∴ Overall incidence of B+ve cells	:	31% (151 / 488)
Incidence of colonies containing B+ve cells	:	96% (22 / 23)
Mean no.of cells per colony (mean± SD)	:	21 ± 62
Median " " "	:	4
Clonogenicity (60 wells ≡ 30 cells)	:	77% (23 / 30)

Table 17G. **48 hr exposure**

Clone #	Total no. of cells	No. of B+ve cells
1	2	2 (≡100%)
2	6	6 "
3	2	2 "
4	2	2 "
5	4	4 "
6	2	2 "
7	2	2 "
8	2	2 "
9	3	3 "
Total no. of cells	**25**	**25**

∴ Overall incidence of B+ve cells : 100% (25 / 25)

Incidence of colonies containing B+ve cells : 100% (9 / 9)

Mean no.of cells per colony (mean ± SD) : 2.8 ± 1.4

Median " " " : 2

Clonogenicity (60 wells ≡ 30 cells) : 30% (9/30)

Table 17 Summary

Exposure Time (hr)	Total no. of cells	No. of B+ve cells	% B+ve	Incidence of cols containing B+ve cells (%)	Median no.of cells per colony	Cloning Efficiency (%)
0	12,650	110	0.87	21	~1,000	47
4	16,000	114	0.71	22	~1,000	60
8	10,000	210	2.1	56	~625	60
16	4,500	257	4.4	68	156	63
24	3,000	172	5.7	78	50	60
32	488	151	31	96	4	77
48	25	25	100	100	2	30

The overall incidence of B+ve cells was calculated as before. The data clearly demonstrate that REL-C7 cells can indeed become **committed** to differentiate (Table 17 Summary). Exposure to DMSO for 4 hr did not induce significant differentiation when compared to control cells. The overall proportion of B+ve cells after 4 hr DMSO exposure was 0.71%, compared to 0.89% in control cultures. However a marked increase in the percentage of B+ve cells was observed in those cells exposed to DMSO for 8 hr: 2.1% B+ve cf 0.89% in control cells. The proportion of cells that stained B+ve continued to rise with increasing exposure to DMSO:- 16 hr exposure, 4.4 % B+ve; 24 hr exposure, 5.7% B+ve; 32 hr exposure, 31% B+ve; and 48 hr exposure, 100% B+ve.

Thus, data are presented which demonstrate that DMSO is not required to be present continually to induce differentiation in REL-C7 cells. Furthermore, although it may take cells some 96 hr in conventional suspension cultures to achieve extensive accumulation of haemoglobin, a proportion of REL-C7 cells can be committed to differentiate after as little as 8 hr exposure to DMSO. With increasing exposure times, the proportion of cells that become committed correspondingly increases.

3.5.4 Phenotypic analyses of REL-C7 colonies following incubation with DMSO

When examining colonies for the presence of B+ve cells, three types of colony could be defined:-

a) undifferentiated colonies, i.e. containing no B+ve cells,

b) mixed colonies, i.e. containing both B+ve and B-cells,

c) differentiated colonies, i.e. exclusively B+ve.

These data are summarised in Table 18.

Control cultures (i.e. no exposure to DMSO) and cells exposed to DMSO for 4 hr produced very similar results. From these cultures, the majority of colonies obtained were undifferentiated and exclusively B-ve. At the time of assessment (120

Table 18

Heterogeneity of clones from REL-C7 cells exposed to 1.5% DMSO for various periods of time.

Exposure Time (hr)	Undifferentiated Colonies (%)	Mixed Colonies (%)	Uniformly B+ve Colonies (%)
0	79	21	0
4	78	22	0
8	44	56	0
16	32	58	11
24	22	44	33
32	4	22	74
48	0	0	100

hr of culture), these colonies contained of the order of 1,000 cells. The rest of the colonies (0 exposure, 21%, 4 hr exposure 22%) were of the mixed phenotype. These mixed colonies were generally of comparable size with the undifferentiated colonies although a small proportion contained only 50-500 cells. With the exception of clone #9 from cells exposed to DMSO for 4 hr, these mixed colonies contained 0.8 - 12% differentiated cells. These differentiated cells tended to be present in small local foci within the colony. No uniformly B+ve colonies were detected.

After 8 hr exposure to DMSO, a marked alteration had taken place in the proportions of the various colony types. Less than half (44%) of the colonies were now of the undifferentiated type. Although some colonies still contained ~1,000 cells, 50% of the colonies were smaller, containing 32- approximately 250 cells. The majority (56%) of colonies were mixed. The proportion of differentiated cells within any one colony ranged from 0.5 - 27%. The colony size was fairly heterogeneous, varying from 32 cells up to ~1,000 cells.

After 16 hr exposure to DMSO, a further shift was observed. The proportion of undifferentiated colonies had decreased further to 32%. Again, there was a general reduction in colony size with only 1 out of 19 colonies containing ~1,000 cells. The majority (58%) of colonies was mixed with the proportion of differentiated cells in the range 2-50% of any one colony. Interestingly, with this exposure time, a small (2 out of 19) but significant proportion of colonies were exclusively B+ve. These colonies were small and compact and contained 16 and 10 cells respectively.

A further change was observed with those cells exposed to DMSO for 24 hr. The proportion of undifferentiated colonies showed a further decrease from 32% (16 hr exposure) to 22%. In addition, the proportion of mixed colonies also decreased from 58% (16hr exposure) to 44%. This fall could be accounted for by the marked increase (3-fold) in the proportion of colonies that were uniformly B+ve from 11% (16 hr exposure) to 33%. These colonies were small, containing 3-8 cells per colony.

This trend continued with 32 hr DMSO exposure. As the proportion of undifferentiated colonies and mixed colonies decreased (22 → 4% and 44 →22%

respectively), the proportion of fully differentiated colonies continued to increase. In fact, of all the colonies obtained, 75% were uniformly B+ve. These colonies rarely contained more than 8 cells per colony (the exception was colony # 14 which contained 14 cells).

By 48 hr of DMSO exposure, all the colonies that were detected were uniformly B+ve. These "colonies" were small, containing between 2 and 6 cells per colony. Only 9 out of 30 wells were positive for cell growth. Clearly, this number is small. If more cloned cells had been analysed, it is likely that some mixed colonies would have been observed i.e. some cells that had not fully differentiated.

This analysis of colony phenotype clearly demonstrates the effects of DMSO on individual cells. In control cells (and even 4 hr DMSO exposure cells) it can be seen that most cells produce progeny that are undifferentiated. A minority of these control cells produce progeny that contain a small proportion of differentiated cells. With increasing exposure to DMSO, a pattern emerged whereby more and more individual cells started to produce progeny with an increasing proportion of differentiated cells. This continued until the vast majority of progeny cells were differentiated, illustrating the process of commitment of differentiation.

3.5.5. ^{3}H Thymidine incorportion.

It has been amply demonstrated that exposure to DMSO induces REL-C7 cells to differentiate and down-regulates proliferation as assessed by cell division. Clearly, cell division is the culmination of many diverse cellular acitivities working in concert. Rather than look at this complex process, experiments were set up to look at one aspect of cell division, namely DNA synthesis.

Multiple cultures of REL-C7 cells were exposed to 1.5 % DMSO for varying periods of time. Proliferation was then assessed by adding to the cultures, a pulse of radioactive label (^{3}H-thymidine) (see Section 2.10). Thus, those cells that are proliferating rapidly will incorporate relatively large amounts of ^{3}H-thymidine, while those cells in which proliferation has been down-regulated will incorporate

correspondingly low levels of the label. This represents a very sensitive technique for detecting subtle changes in the proliferative status of cells.

In initial experiments, REL-C7 cells were exposed to DMSO for 24 hr, 48 hr, 72 hr and 96hr. At the various time points when cells were set up in DMSO-containing cultures, control cultures were also established i.e. DMSO free cultures. Eighteen hours before the end of the experiment, the cells from each flask culture were spun down and resuspended at 0.05 x 10^6/ml. Aliquots of 200 µl were dispensed into the wells of a tissue culture plate, and the ^{3}H-thymidine was added. Thus, for each DMSO time point, the same number of cells was assessed in the thymidine incorporation assay. This allowed direct comparison between the cultures from various time points. Data from three experiments are summarised in Table 19.

In all three experiments, exposure to DMSO resulted in a progressive reduction in cell proliferation. In two of the experiments (Experiments # 1 and #2), a significant reduction in proliferation was obtained after 24 hr exposure to DMSO as compared to control cultures which were not exposed to DMSO at all (in both cases, $p<0.02$; student's t-test). This proliferative down-regulation became more pronounced with increasing exposure time to DMSO, and in subsequent cultures (i.e. 48 hr, 72 hr, and 96 hr exposure) this down-regulation became highly significant ($p<0.001$).

Experiment #3 showed the same general effects as #1 and #2. As above, after 24 hr exposure to DMSO, there was a reduction in cell proliferation, but on this occasion it was not significant ($0.05<p<0.1$). However, after 48hr exposure there was a significant reduction in proliferation ($p<0.01$) and this reduction became highly significant after 72 hr and 96 hr exposure ($p<0.001$). To ensure that any reduction in proliferation was due to DMSO and not due to any limitation in the culture conditions (e.g exhaustion of nutrients, accumulation of cellular waste products), control cultures containing no DMSO were set up in parallel. These cells were manipulated in the exact same fashion as the DMSO cultures, i.e. at the appropriate time, the flask cultures were poured into a centrifuge tube, spun down, resuspended, the cell count was adjusted and aliquots of 200µl were dispensed into the wells of a 96-well tissue

Table 19

Effect of DMSO exposure on the uptake of ^{3}H-thymidine into REL-C7 cells.

Experiment 1

DMSO Exposure (hr)	n	mean ± SD	P-value
0	9	23977 ± 2583	-
24	9	19298 ±3334	P < 0.02
48	10	12772 ± 858	P < 0.001
72	10	9211 ± 1046	P < 0.001
96	10	5507 ± 1315	P < 0.001

Values are counts per minute (cpm).

Experiment 2

DMSO Exposure (hr)	n	mean ± SD	P-value
0	8	35258 ± 2661	-
24	10	31157 ± 1907	P < 0.02
48	10	22919 ± 2006	P < 0.001
72	9	13574 ± 3885	P < 0.001
96	10	9293 ± 964	P < 0.001

Experiment 3

DMSO Exposure (hr)	n	mean ± SD	P-value
0	10	22552 ± 4406	-
24	10	18421 ± 2808	0.05 < P <0.1
48	10	16002 ± 1655	P < 0.01
72	10	5866 ± 566	P < 0.001
96	10	5094 ± 453	P < 0.001

culture plate. Data are presented in Table 20 which indicate that all these cultures retained similar levels of proliferation. There were no significant differences between any of these control cultures. Thus, any reduction in proliferation is unlikely to be due to non-specific culture effects, but is most likely to be due to a proliferative down-regulation induced by DMSO.

Although each of these experiments showed the same down-regulatory effect of DMSO, there was wide variation in the raw data generated (i.e. cpm) between experiments. For example, in experiments # 1 and #3, the greatest uptake of ^{3}H-thymidine was seen, as expected, in the cells that were cultured in the absence of DMSO; 23, 977 cpm and 22,552 cpm respectively. However, in experiment #2, the corresponding DMSO-free culture generated 35,258 cpm, about half as much again compared to experiments #1 and #3. Indeed, in experiment #2, cells exposed to DMSO for 48 hr showed a highly significant ($p<0.001$) reduction in proliferation as compared to control cultures. Yet these cells exposed to DMSO for 48 hr generated 22,919 cpm - very similar to the control cultures in experiments #1 and #3. To combine the results of the experiments, it was felt necessary to "normalise" the data within each experiment, i.e. in a given experiment the cpm from the control (no DMSO) culture was deemed to be 100%, and the subsequent cpm's from the DMSO-treated cultures were expressed as a percentage of the control (Table 21). Thus, although between experiments there is some variability in the data, within each experiment the data is very reproducible (note the "tight" value for the SEM's).

The data from Table 21 may be represented diagramatically (Figure 11). A linear relationship exists between length of exposure to DMSO and percentage decrease in ^{3}H-thymidine uptake (proliferation).

Clearly, then, over a period of time, DMSO can exert a powerful anti-proliferative influence on REL-C7 cells. The linear relationship demonstrated in Figure 11 implies an early onset of this down-regulatory effect. In an effort to see just how rapidly DMSO can induce this proliferative switch-off, further experiments were set up in which REL-C7 cells were exposed to DMSO for shorter periods of time, viz,

Table 20

Uptake of ^{3}H-thymidine into REL-C7 cells. Control cultures for REL-C7 cells exposed to DMSO for various periods of time.

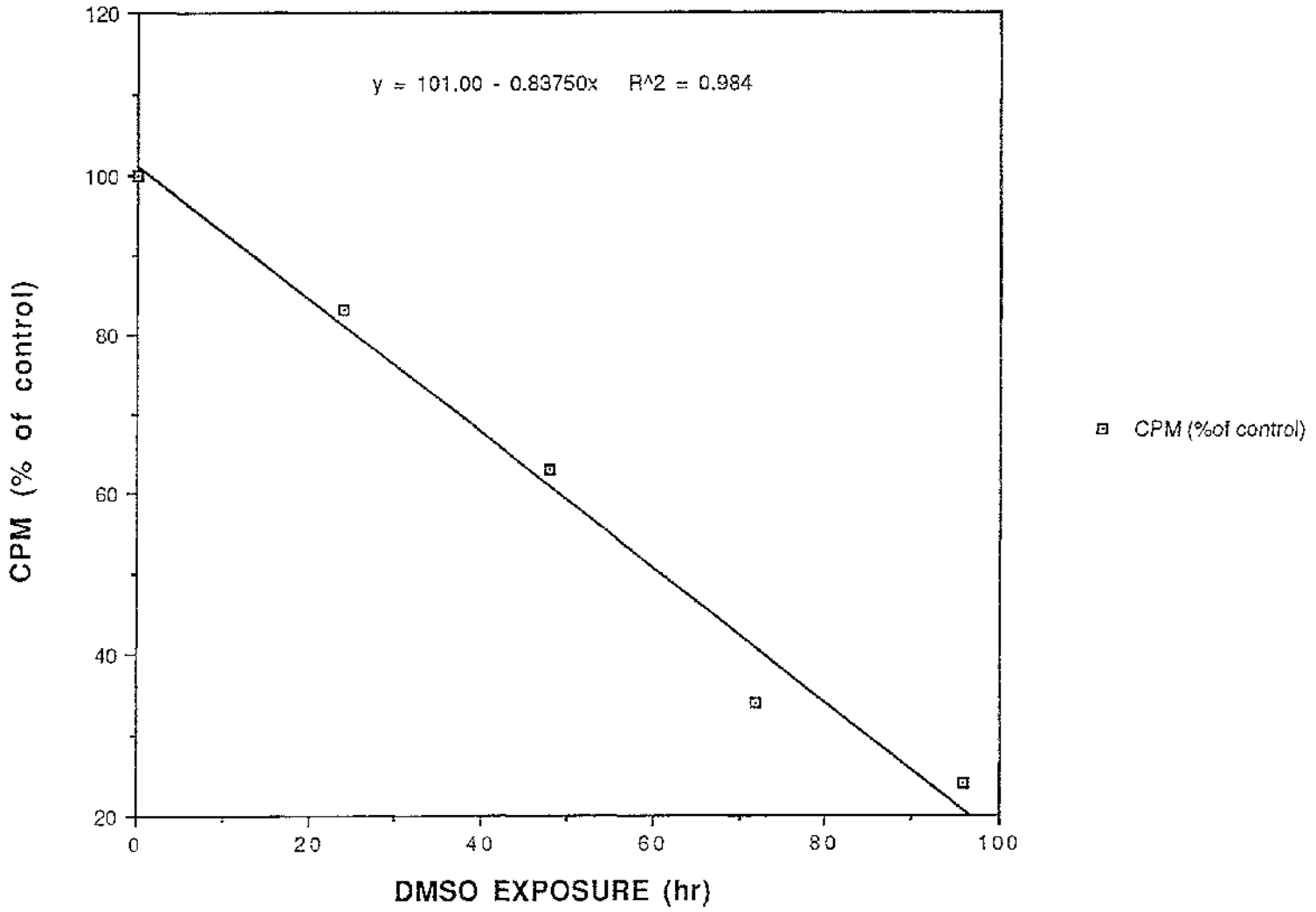
Duration of culture (hr)	n	mean ± SD	P-value
24	10	35258 ± 2661	-
48	10	34969 ± 1603	$0.5 < P$ NS
72	10	38092 ± 5548	$0.1 < P < 0.5$ NS
96	10	32071 ± 3864	$0.1 < P < 0.5$ NS

Table 21

Effect of DMSO on ^{3}H-thymidine uptake by REL-C7 cells. Data expressed as percentage of control cultures.

DMSO Exposure (hr)	cpm (% of control)	mean ± SEM
0	100	-
24 : #1	80	
#2	88	83 ± 2.4
#3	82	
48 : #1	53	
#2	65	63 ± 5.3
#3	71	
72 : #1	38	
#2	38	34 ± 4.0
#3	26	
96 : #1	23	
#2	26	24 ± 1.0
#3	23	

Fig.11
Incorporation of tritiated thymidine into REL-C7 cells exposed to 1.5% DMSO.



2 hr, 4 hr, 6 hr and 8 hr. The methodological details are laid out in Section 2.10. Data from three experiments are summarised in Table 22.

These data corroborate the previous studies, in that increasing exposure to DMSO progressively reduces cell proliferation as measured by ^{3}H-thymidine uptake. In all three experiments there was a detectable reduction in proliferation after as little as 2 hr exposure to DMSO. However, there was wide variation in the statistical significance of these changes. The difference was significant at the level of $p<0.05$ in experiment #1 and significant at the level of $p<0.01$ in experiment #2. However, in experiment #3, the small reduction after 2 hr exposure to DMSO was not significant ($p>0.5$). After 4 hr exposure, the proliferative down-regulation was highly significant ($p<0.001$) in experiments #1 and #3. In experiment 2 the results were less clear cut. The cpm value after 4 hr exposure was actually slightly higher than the figure for 2 hr exposure, though not significantly so ($p>0.5$). Compared to the control value, the 4 hr exposure value was still statistically significantly different ($p<0.05$; cf 2 hr exposure, $p<0.01$).

These minor discrepancies probably reflect the limits of the sensitivity of the ^{3}H-thymidine uptake assay. When there are only small, subtle differences between test samples, it becomes difficult to demonstrate any reproducible, statistically significant changes.

In any event, all three experiments demonstrate a highly significant ($p<0.001$) reduction in proliferation after 6 hr exposure to DMSO as compared to control cultures, and at least a significant ($p<0.05$) reduction after 4 hr exposure.

These data clearly demonstrate that DMSO can rapidly modulate the proliferative status of REL-C7 cells. This effect can be first detected after 2-4 hr exposure to DMSO. Previously, (Section 3.5.3., DMSO-induced erythroid differentiation) it was shown that REL-C7 could become committed to differentiate after 8 hr exposure to DMSO. It is likely that this reduction in the rate of proliferation is a necessary early event in the process of commitment to differentiation.

Table 22

Effect of DMSO Exposure on the uptake of ^{3}H-thymidine into REL-C7 cells.

Experiment 1

DMSO Exposure (hr)	n	mean ± SD	P-value
0	10	8816 ± 747	-
2	10	7667 ± 1479	$P < 0.05$
4	10	6218 ± 1247	$P < 0.001$
6	10	5614 ± 1252	$P < 0.001$
8	10	5531 ± 1319	$P < 0.001$

Values are cpm.

Experiment 2

DMSO Exposure (hr)	n	mean ± SD	P-value
0	10	2913 ± 103	-
2	10	2431 ± 30.2	$P < 0.01$
4	9	2486 ± 64.4	$P < 0.05$
6	10	1836 ± 52.2	$P < 0.001$
8	10	1638 ± 100	$P < 0.001$

Experiment 3

DMSO Exposure (hr)	n	mean ± SD	P-value
0	6	4346 ± 799	
2	6	4072 ± 710	$P > 0.5$ NS
4	6	3394 ± 178	$P < 0.001$
6	6	3158 ± 191	$P < 0.001$
8	6	2250 ± 387	$P < 0.001$

NS = not significant.

3.5.6. Cell Cycle Analyses

In the previous two sections, data were presented showing that as REL-C7 cells became committed to erythroid differentiation, their proliferative ability correspondingly declined. This proliferative down-regulation was measured by colony formation (*in vitro* clonogenic assay) and ^{3}H-thymidine incorporation. In this section, experiments were set up to analyse further the way in which DMSO effected these profound proliferative changes. Cell cycle analyses were performed on REL cells that had been exposed to DMSO for various periods of time.

In eukaryotic cells, DNA synthesis does not occur throughout the cell cycle, but is restricted to a special phase of it, occurring before mitosis (cell division). This phase is called S-phase (S for synthetic). A gap of time occurs after DNA synthesis and before cell division. Another time gap occurs after division and before the next S-phase The cell cycle, thus consists of the M (mitotic) - phase, a G_1-phase (the first gap), the S-phase, a G_2 phase (the second gap), and back to M. Some non dividing cells can suspend the cell cycle after mitosis and just prior to DNA synthesis. Such "resting" cells, which have exited from the cell cycle before the S-phase, are said to be in the G_0 state.

The cell cycle status of the various cell suspensions was assessed by measuring the uptake and incorporation into DNA of a thymidine analogue, bromodeoxyuridine (BrdU) (Section 2.11). Thus, when cells were pulse labelled with BrdU, only those cells which were actively synthesising DNA (i.e. in the S-phase of the cell cycle) could incorporate BrdU into their DNA. A monoclonal antibody, anti-BrdU, was then used to identify those cells undergoing DNA synthesis at the time of the pulse. The proportion of cells in S-phase of the cell cycle was then determined by flow cytometry. In addition, the cell suspensions were incubated with propidium iodide (PI) which stains DNA. Thus, the total DNA content of cells could be assessed by measuring PI uptake. In this manner, it was possible to identify three populations of cells depending on their position in the cell cycle. Gates were then drawn round each of these subpopulations and by counting the number of events within each

defined area, the percentage of cells in each phase of the cell cycle could be determined. A specimen contour plot from the Becton Dickinson Source Book is shown in Figure 12.

Those cells which are quiescent (i.e. G_0) or have just completed mitosis (i.e. in G_1) will not be synthesising DNA, will therefore not have incorporated significant amounts of BrdU into their DNA and therefore will show no positive reaction with the FITC-labelled anti-BrdU. They will each contain "n" amount of DNA, where n is an indeterminate unit value.

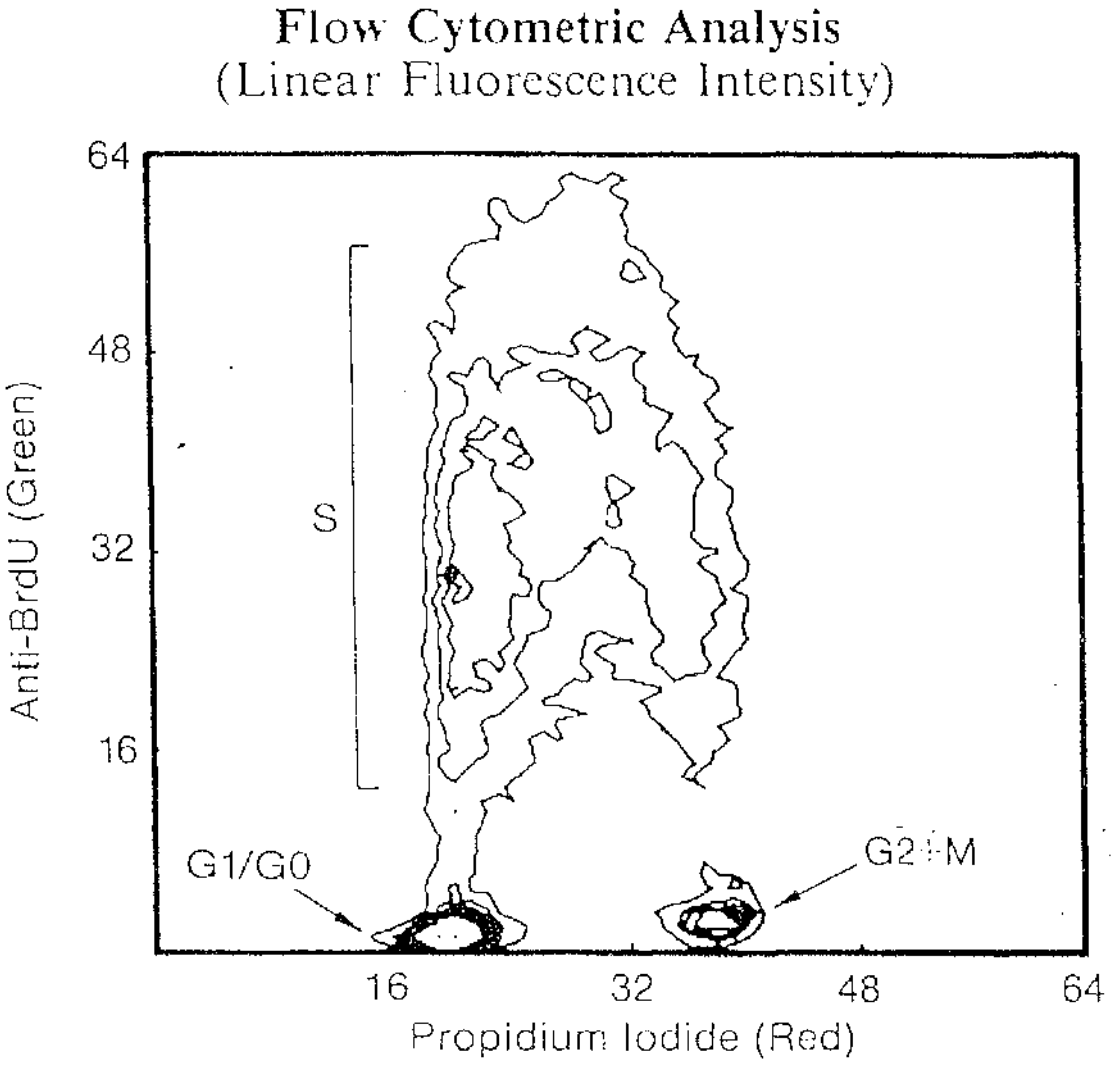
Those cells which have entered S-phase will be actively synthesising DNA, will therefore have incorporated BrdU into their DNA and will show a positive reaction with anti-BrdU-FITC. There will be some variation in the amount of BrdU incorporated by the cells in this subpopulation. This reflects the level of activity of DNA synthesis in individual cells over the time period when BrdU was available to be incorporated. Thus, those cells that were already in S-phase when the 30 minute incubation with BrdU was started, would be able to incorporate BrdU over the entire 30 minutes exposure (presuming S-phase extends for more than 30 minutes) and would tend to accumulate relatively large amounts. Conversely, those cells that perhaps entered S-phase at some point during the BrdU incubation would be able to incorporate BrdU for only a corresponding proportion of that 30 minute period. They would, therefore, accumulate less BrdU.

Lastly, those cells that had completed S-phase would now be in G_2 or undergoing mitosis (M). As in the first subpopulation (G_0/G_1), the cells in G_2+M would not be synthesising DNA, and therefore would incorporate insignificant amounts of BrdU. However, in contrast to the cells in G_0/G_1, the cells in G_2+M would be either in mitosis or preparing for it. Consequently, prior to cell division, by which each of the two daughter cells receives the full complement of genetic material, these cells in G_2+M will contain twice the amount of DNA, i.e. "2n". So although the cells in G_0/G_1 and the cells in G_2+M cannot be distinguished on the basis of BrdU

Figure 12

Specimen contour plot from FACScan showing the various phases of the cell cycle.

(Taken from the Becton Dickinson Source Book)



uptake (since neither incorporates it), they can be resolved by the amount of PI staining which is proportional to the total DNA content.

The data from two experiments are presented in Table 23. These data were generated by analysing the various cell suspensions with the Consort software. An example is shown in Figure 13.

Both experiments demonstrate two main alterations to the cell cycle status of REL-C7 cells exposed to DMSO. Firstly, there is a decrease in the proportion of cells in S-phase, i.e. synthesising DNA. Secondly, there is an increase in the proportion of cells in G_0/G_1. Between experiments, there appears to be some discrepancy in the actual numerical values obtained. The method used to prepare the cells for analysis is quite elaborate, requiring a lot of cell manipulation and processing. In addition, when the samples are actually analysed by flow cytometry, some subjective decisions have to be made, e.g. where to position the gates; in which defined area should particular events be included. These aspects of the method will introduce inaccuracies and make reproducibility between experiments rather difficult to achieve. In any event, individually, each experiment demonstrates that as the proportion of cells in S-phase declines, there is a corresponding increase in the proportion of cells in G_0/G_1 phase. As an aid to following these changes in cell cycle status, these data have been represented diagramatically (Figures 14A, 14B).

In experiment #1, the first appreciable shift was detected between 4 and 6 hr of DMSO exposure. The proportion of cells in S-phase dropped from 58% to 41% and the decline continued to 35% (8 hr exposure) and to 29% (24 hr exposure). Correspondingly, the proportion of cells in G_0/G_1 increased from 30% (4 hr) to 47% (6 hr) and this accumulation of cells in $G_0/G1_1$, continued to 54% (8 hr) and to 60% (24 hr). Over the exposure times studied, the proportion of cells in G_2+M remained largely unaltered, ranging from 8-14%. However, there was perhaps some evidence for changes in the level of G_2+M cells. Over the earlier time points the proportion of cells in G_2+M rose from 8% to 12% (1 hr) to 14% (2 hr and 3 hr) before declining to 12% (4 hr and 6 hr) and to 10% (8 hr). These changes were, to say the least, modest

Table 23

Effect of DMSO on the cell cycling status of REL-C7 cells. Percentage of REL-C7 cells in the various phases of the cell cycle.

A. Experiment 1

DMSO Exposure (hr)	G_0 / G_1	S-phase	G_2 + M
Control	32	58	10
0	23	69	8
1	29	59	12
2	24	62	14
3	29	57	14
4	30	58	12
6	47	41	12
8	54	35	10
24	60	29	11

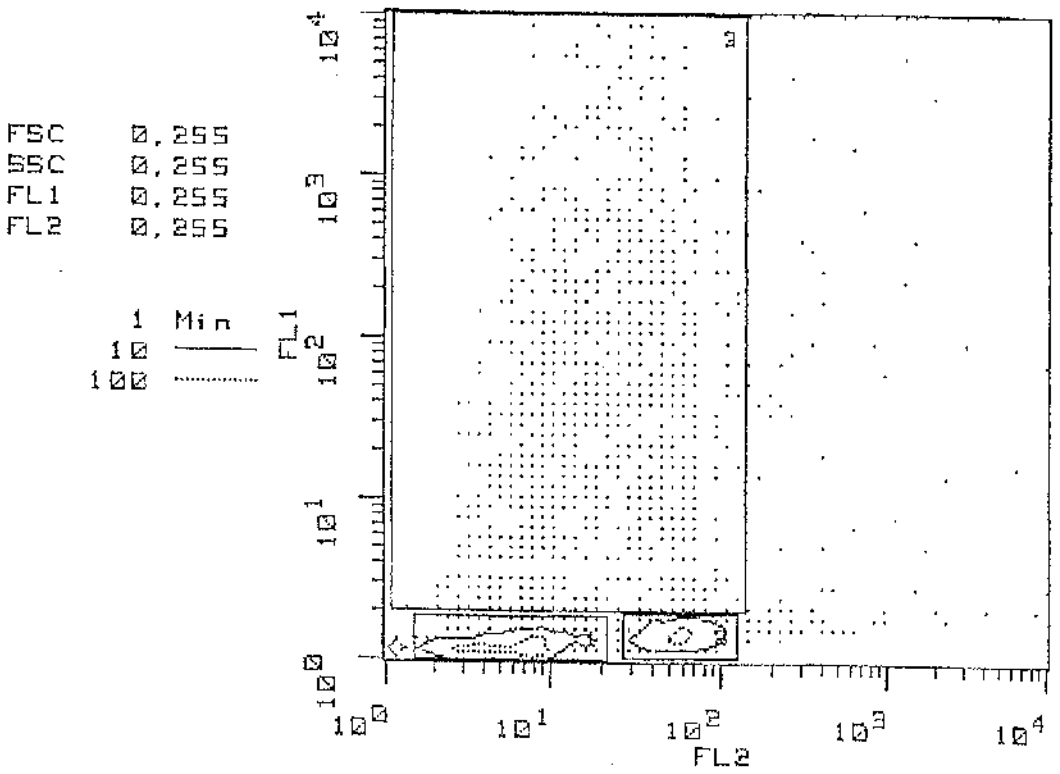
B. Experiment 2

DMSO Exposure (hr)	G_0 / G_1	S-phase	G_2 + M
Control	15	76	9
0	11	83	7
1	15	76	9
2	22	46	32
3	26	36	38
4	50	27	23
6	62	23	14
8	68	23	8
24	69	27	4

Figure 13

Contour plot of REL-C7 cells exposed to DMSO for 4 hours.

See text for method and interpretation.



Sample : SAMPLE 011
Cytometer: FACSCAN

Contour statistics
Parameters : FL2 FL1 Gated events : 5000

#	X & Y Lower	X & Y Upper	Events	% Gated	% Tot
1	1.55 1.00	21.54 1.79	2428	48.56	48.56
2	28.86 1.16	124.52 1.79	1134	22.68	22.68
3	1.16 2.08	124.52 10000	1280	25.60	25.60

Figure 14A **Experiment 1: Percentage of REL-C7 cells in various phases of the cell cycle, following exposure to 1.5% DMSO.**

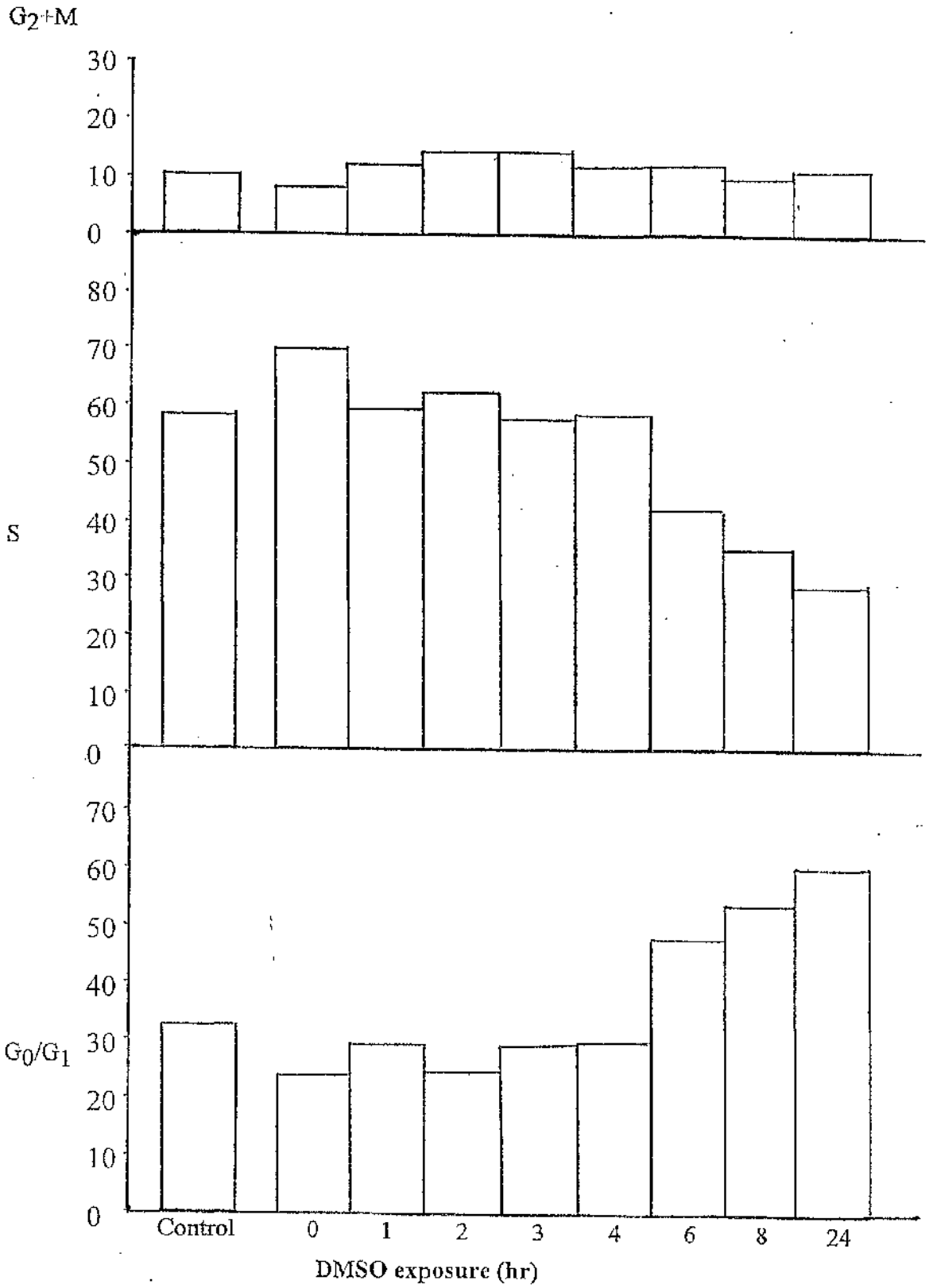
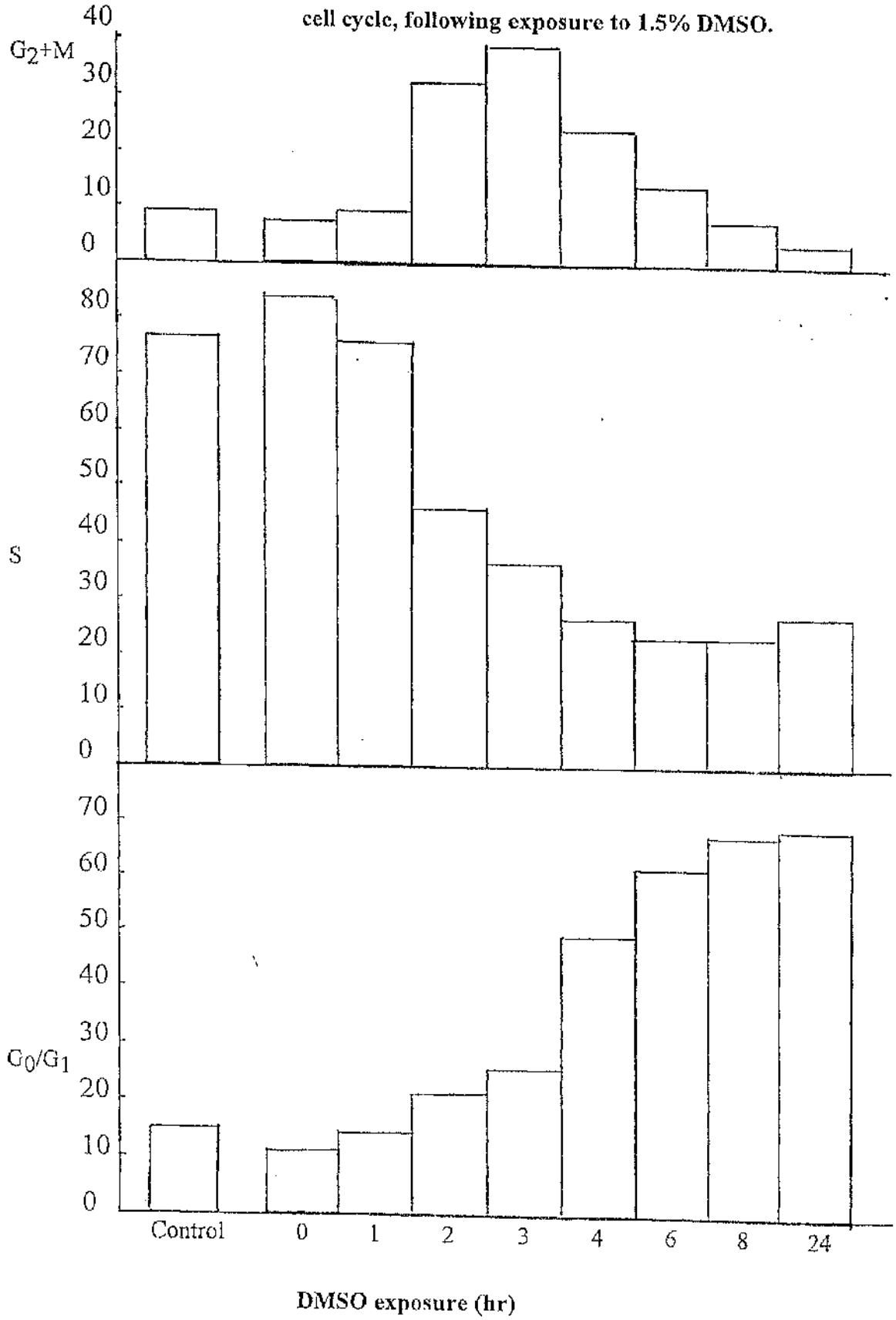


Figure 14B **Experiment 2: Percentage of REL-C7 cells in various phases of the cell cycle, following exposure to 1.5% DMSO.**



and certainly did not provide convincing evidence of significant fluctuations in the levels of G_2+ M cells. The major alterations were found in the levels of G_0/G_1 cells and S-phase cells, and indeed, the changes tended to occur in a reciprocal fashion, i.e. as the proportion of cells in S-phase declined, then the proportion of cells in G_0/G_1 increased.

The same general pattern was demonstrated in experiment #2 and, in fact, the alterations in the various phases of the cell cycle were more pronounced. Again, as the proportion of cells in S-phase declined, then the proportion of cells in G_0/G_1 correspondingly increased. The first appreciable shift in S-phase cells was detected between 1 and 2 hr of DMSO exposure with a drop from 76% to 46%. This trend continued over the following time points: - 36% (3 hr) to 27% (4 hr) to 23% (6 hr) and more or less maintained at this level: 23% (8 hr) and 27% (24 hr).

As the proportion of cells in S-phase declined, the proportion in G_0/G_1, steadily rose with increasing exposure to DMSO. Small, but detectable, alterations were seen even at the shortest DMSO exposure times. After 1 hr exposure to DMSO, the proportion of cells in G_0/G_1 rose from 11% to 15% and subsequently to 22% (2 hr) and to 26% (3 hr). Between 3 hr and 4 hr exposure to DMSO, there was a marked elevation in G_0/G_1 cells from 26% to 50% and this trend continued to 62% (6 hr) to 68% (8 hr) to 69% (24 hr).

Although in experiment #1 there were only marginal alterations in the levels of G_2+M cells, in experiment #2 changes were far more obvious. Between 1 hr and 2 hr exposure to DMSO, the proportion of cells in G_2 + M rose markedly from 9% to 32%. This rise continued to 38% with 3 hr DMSO exposure and then started to decline to 23% (4 hr) to 24% (6 hr) and back to baseline with 8% (8 hr) and 4% (24 hr).

Data are presented which demonstrate that relatively brief exposure to DMSO can bring about profound alterations in the cell cycling status of REL cells. After between 4 and 6 hr exposure to DMSO a marked increase was detected in the proportion of cells in G_0/G_1. Over further time points, cells continued to accumulate

in G_0/G_1 reaching a maximum of 69%. This rise in G_0/G_1 cells can be accounted for, mainly, by a fall in the proportion of cells in S-phase. In one of the experiments, this decrease in S-phase cells coincided with the increase in G_0/G_1 cells, the most dramatic changes occurring after 4-6 hr of DMSO exposure. In the other experiment, the same general trend was obtained although the inter-relationships between the various phases of the cell cycle were less clear cut. There was a marked decline in S-phase cells after 1-2 hr DMSO exposure. This was accompanied by a 3.6 - fold increase in G_2+M cells and a more modest increase in G_0/G_1 cells. The proportion of S-phase cells continued to decline and after 3-4 hr DMSO exposure the proportion of G_0/G_1 cells increased markedly from 26% to 50%.

As has been demonstrated here and in previous sections, brief exposure of REL cells to DMSO can commit cells to differentiate with a concomitant down-regulation of proliferation. From growth curves it was shown that the rate of cell division decreases. From analyses of ^{3}H-thymidine incorporation, DNA synthesis was shown to be switched off. Cell cycle studies have indicated that the proportion of cells synthesising DNA declines because cells accumulate in G_0/G_1. They seem to be unable to make the transition from G_1 into S-phase. Clearly, if there is no DNA synthesis, then there can be no cell division. It seems likely that DMSO (and other similar chemical inducers) exerts its effects, not by directly switching off DNA synthesis, but rather by preventing cells from passing from G_1 into S-phase. This cell cycle "block" can be induced rapidly, requiring only a few hours exposure to DMSO.

3.5.7. Assessment of Cell Size

In preceding sections, experiments were set up to monitor the subtle changes that occurred when REL-C7 cells were induced to differentiate. In this (and the following) section, gross morphological aspects of differentiating REL-C7 cells were measured, e.g. cell size.

As outlined in the Materials and Methods (Section 2.12), cell size can be assessed by flow cytometric methods, e.g. FACS analyses. Using a FACS, the

forward light scatter (FLS) properties of cells can be measured and, to all intents and purposes, this is proportional to cell size.

It is possible to measure accurately cell size by comparing the properties of a given cell population to those of reference beads of a defined precise diameter. In these studies, however, it was felt unnecessary to obtain the exact cell size. What was of interest was the relative changes that occurred between routinely proliferating REL-C7 cells and REL-C7 cells that had been exposed to DMSO.

REL-C7 cells were exposed to DMSO (1.5%) for various periods of time and were then analysed by FACS to assess their cell size. To allow direct comparison between individual samples, the histogram profiles were overlaid on one display (Figure 15).

It can be clearly seen that REL-C7 cells exposed to DMSO undergo a reduction in cell size. This reduction was detected after as little as 8 hr exposure to DMSO. A significant decrease in cell size was obtained after 24 hr exposure ($p<0.001$; Kolmogorov-Smirnov Two Sample Test (173)). With increasing exposure time, the cells continued to become smaller.

In Figure 15, the units on the x-axis are merely arbitrary units that do not relate to any absolute measurement. Nevertheless, the scale is proportional i.e. cells which have a peak at "800" are, on average, twice the diameter of cells with a peak at "400". For each cell population, the mean cell size (in arbitrary units) was assessed by estimating the peak value for each curve. Clearly, these values are not precise but at least they demonstrate the shift in cell size:-

DMSO	0	:	795 (arbitrary units)
	8	:	765
	24	:	685
	48	:	660
	72	:	645
	96	:	575

Figure 15

REL-C7 cells exposed to DMSO for various periods of time.

Effects on cell size as assessed by forward light scatter properties.

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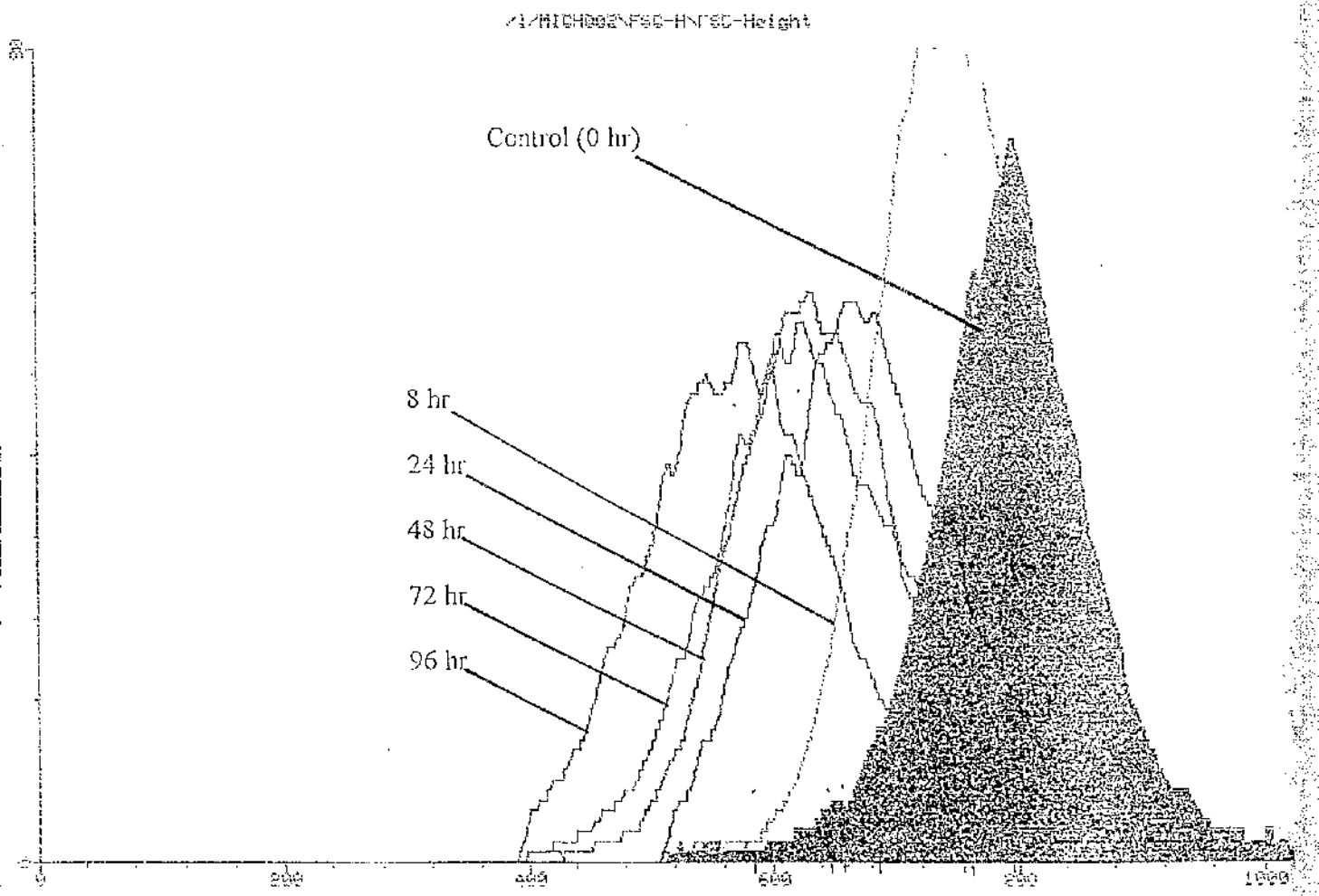


In addition, as would be expected, there is a great deal of overlap between the curves. In any one population, the cells will not be all the one discrete size, but rather there will be a distribution of cell sizes. These histogram profiles show a shift in the size distribution curves. The decrease in cell size can be quite marked. This can be clearly seen by comparing the distributions for 0 hr exposure and 96 hr exposure. There is very little overlap between the two distribution curves, i.e. the vast majority of cells exposed to DMSO for 96 hr are smaller than even the smallest cells in the DMSO 0 hr group.

3.5.8. Scanning Electron Microscopy (SEM)

SEM was kindly provided by Mr J Anderson, Department of Pathology, GRI.

As a further investigation into the morphological changes that may occur when REL-C7 cells are exposed to DMSO, the cell surface morphology was analysed by SEM. As before, REL-C7 cells were incubated with 1.5% DMSO for different periods of time; in this case 24 and 48 hr. The cells were prepared for SEM as described in Section 2.13.

Proliferating REL-C7 cells, cultured in MEM-O++ alone, taken from the logarithmic phase of growth, served as control cells. The cell surface had a convoluted appearance (Plate 5A) and revealed many well defined microvilli and some longer string - like projections. Some short ridges appeared to run across the cell surface.

After 24 hr exposure to DMSO, some profound differences could be seen (Plate 5B). Some cells were similar to the controls. They still retained the rough, uneven surface with ridges running across the cell. However, other cells, with a much smoother morphology, were observed. These cells lacked the microvilli and ridges of control cells. The prominent feature of these cells was the appearances of pores or channels on the cell surface.

After 48 hr exposure to DMSO, a further shift in morphology was detected. Almost exclusively, all the cells observed showed the very smooth morphology. A

representative sample is shown is shown in Plate 5C. Some pores/channels were still discernible.

In previous sections, data have been presented showing that exposure of REL-C7 cells to DMSO causes a down-regulation of DNA synthesis and a reduction in cell proliferation. A further investigation was set up whereby REL-C7 cells were taken from cultures in the stationary phase of growth, i.e. non-proliferating. These cells were cultured in the absence of any inducer, but were allowed to grow and divide until they reached the maximum cell density (approx 3 x 10^6/ml) as shown in earlier growth curves.

These uninduced, non-proliferating cells were analysed by SEM (Plate 5D). Interestingly, they too had a generally smooth appearance with some deep pores/channels on the surface. There was a striking similarity between these cells and the smooth cells observed after 24 and 48 hr exposure to DMSO.

Although differentiation and proliferation seem to be closely related in an inverse fashion, the profound alterations to surface morphology induced by DMSO may reflect proliferation being down-regulated and the cells entering a proliferative stationary phase, rather than any great maturational progression.

It is also worth noting that the complete transformation of the cell surface from rough to smooth, can be effected within 24 hr of exposure to DMSO.

The data presented here also provide some corroboration of the results from the previous section where the size analyses were performed. From the scanning electronmicrographs, it was possible to estimate the actual diameter of cells. The non-induced proliferating control cells measured some 11 μm in diameter. Following exposure to DMSO for 24 hr, cell diameter decreased slightly to about 10 μm. Cells that were exposed for 48 h were even smaller, about 8.5 μm in diameter. The non-induced, non-proliferating control cells measured about 9.5 μm in diameter. Although these reductions may sound modest, if the cell volumes are calculated the differences

Plate 5A

Scanning Electron Micrograph of REL-C7 cells taken from the logarithmic phase of growth.

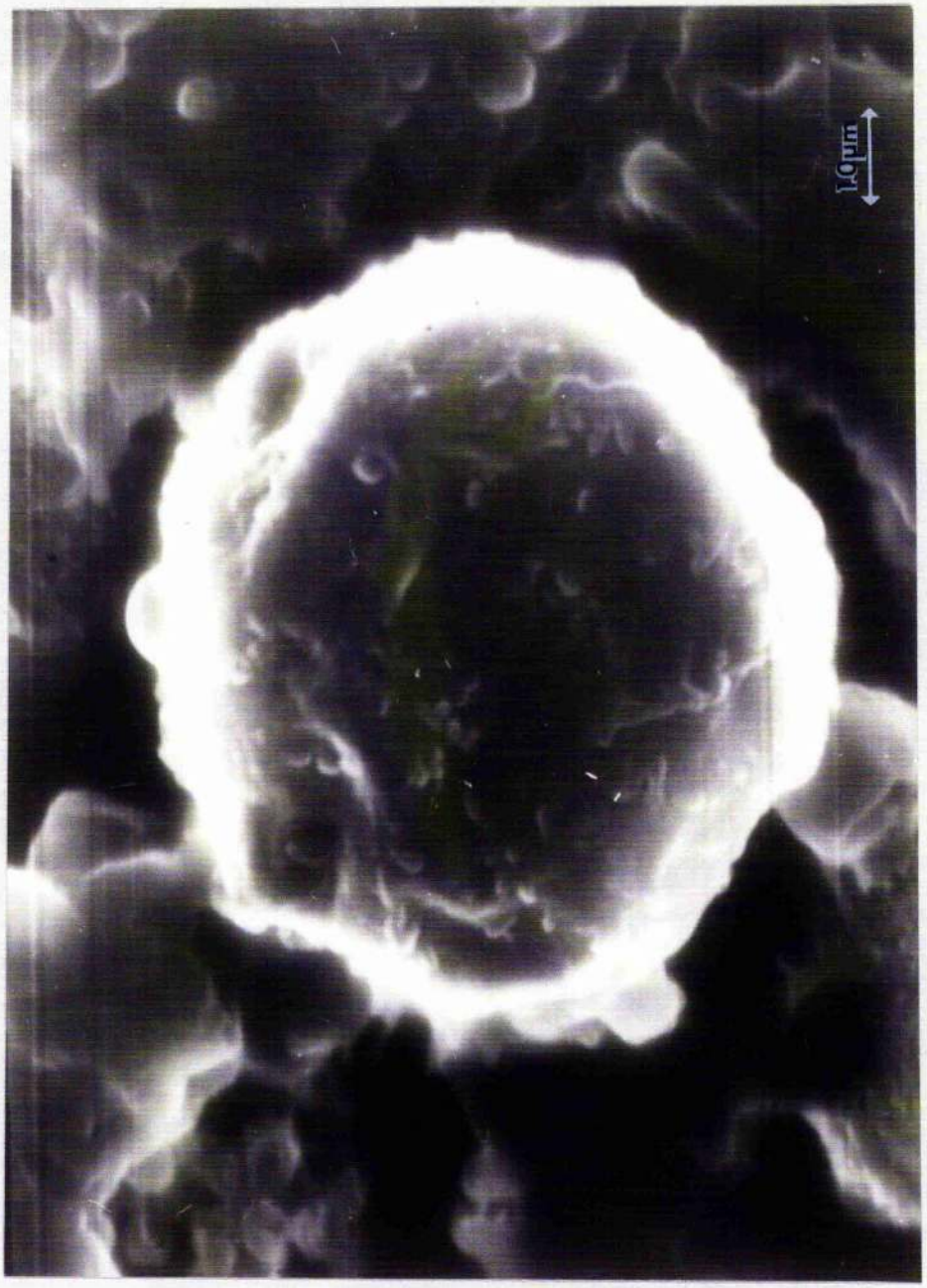


Plate 5B

Scanning Electron Micrograph of REL-C7 cells exposed to 1.5% DMSO for 24 hours.

Plate 5C

Scanning Electron Micrograph of REL-C7 cells exposed to 1.5% DMSO for 48 hours.

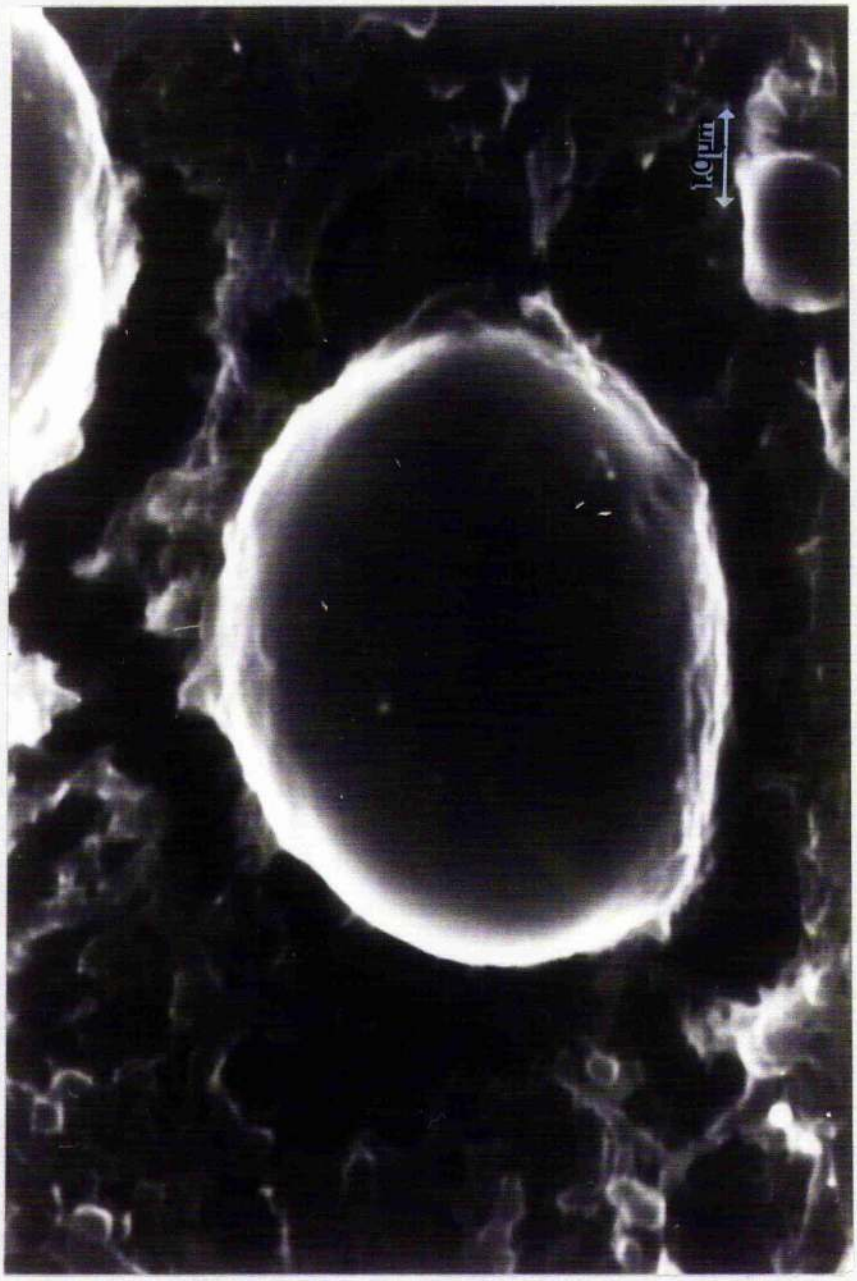
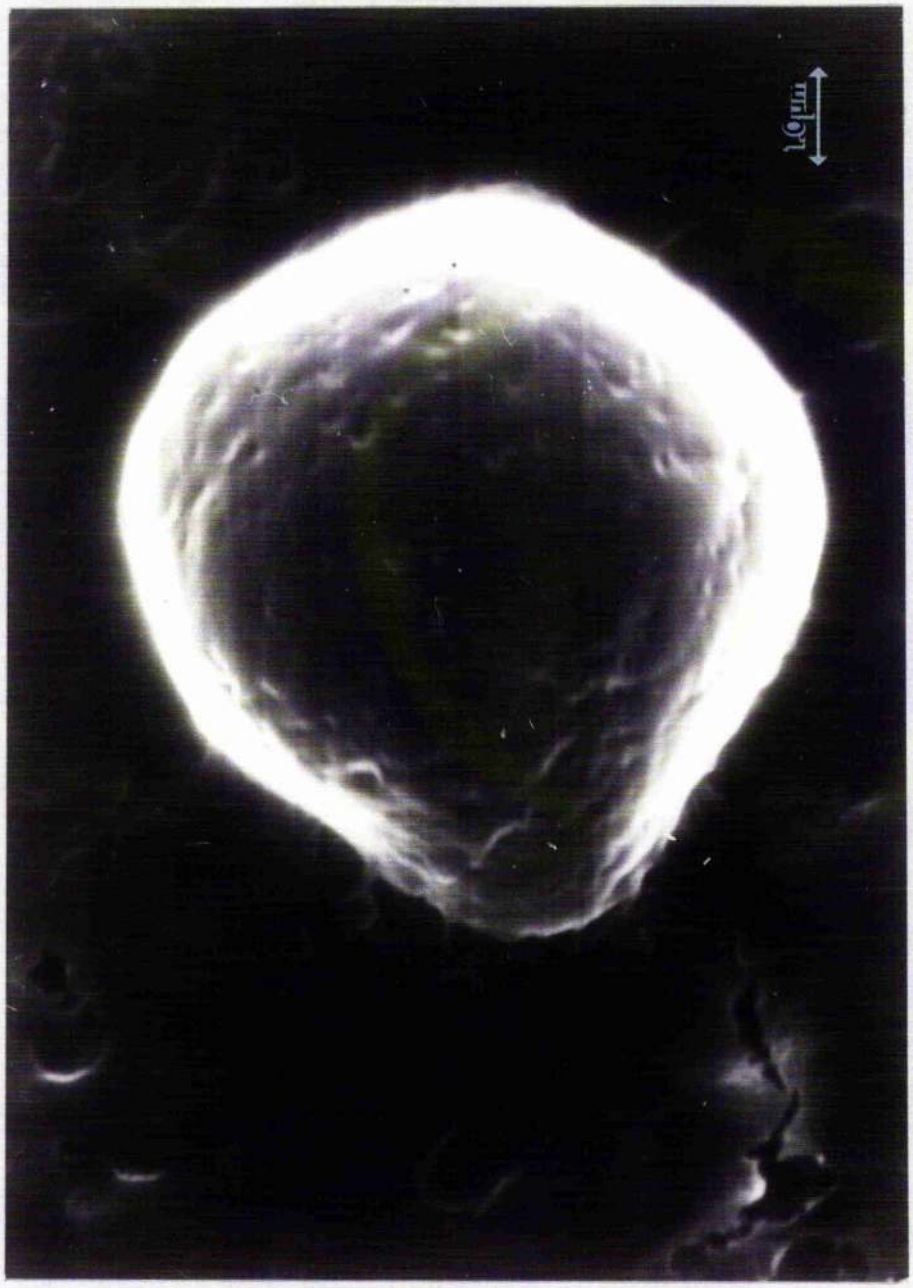


Plate 5D

Scanning Electron Micrograph of non-proliferating REL-C7 cells taken from the stationary phase of growth.



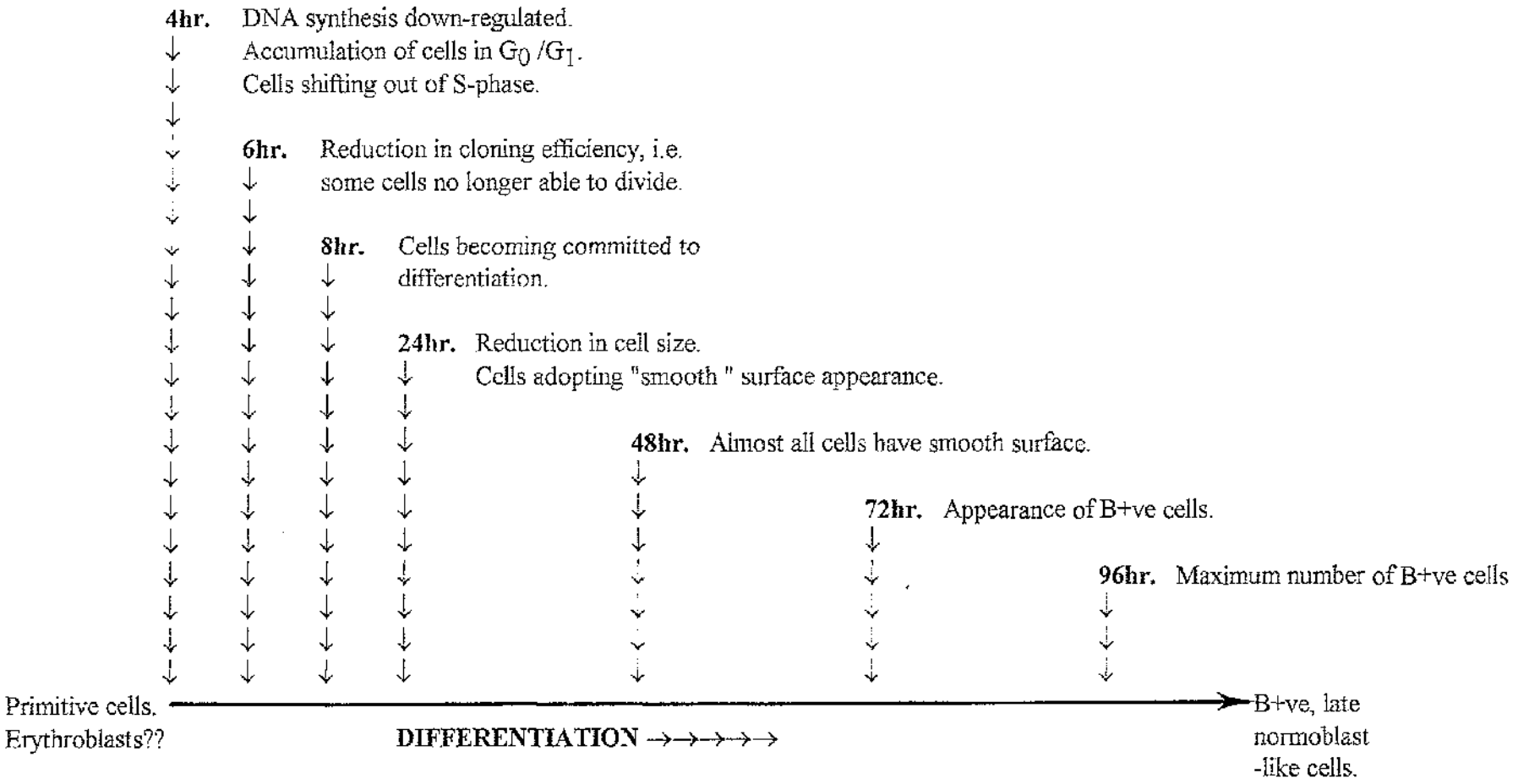
are striking (Presume the cells are spherical; volume of a sphere = 4/3 πr^3, where π is a constant, 3.1416; and r = radius). Using the cell sizes from above, the non-induced proliferating control cells had a volume of 697 μm^3. After 24hr of DMSO exposure, the cell volume had dropped to 524μm^3. After 48 hr of DMSO exposure, the cell volume decreased further to 322 μm^3. For the non-induced, non-proliferating cells, the value was 449 μm^3. Not withstanding the obvious inaccuracies of these calculations, the cell volume appears to be approximately halved following 48 hr exposure to DMSO - a profound reduction by any standards.

In brief summary, than, exposure of REL-C7 cells to 1.5% DMSO seems to induce a cascade of biological effects. It is proposed that, although maximum numbers of B+ve cells are obtained after 96 hr exposure to DMSO, an ordered sequence of events is initiated after only a few hours exposure to DMSO. DNA synthesis is down-regulated and cells start to come out of the proliferative cycle. Cells begin to lose their ability to divide. Some cells become committed to differentiate. Gross morphological changes become apparent, e.g. reduction in cell size, profound alterations to the cell surface whereby the cells change from a rough appearance (by SEM) to a smooth one. Some time later, B+ve cells start to appear and by 96 hr exposure to DMSO, the proportion of B+ve cells is at its maximum. This sequence of events is represented diagrammatically in Figure 16.

3.6 Adaptation of REL-C7 cells to serum-free culture.

In the vast majority of tissue culture techniques, cells are propagated in a chemically defined medium which has been supplemented with serum. The source of serum can vary, but bovine and to a lesser extent, equine are probably the most commonly used. The obvious advantage of such serum supplemented media is that they provide optimum culture conditions for a wide variety of cells. The main disadvantage of these media is that they are chemically ill-defined. Serum constitutes a "soup" containing myriad activities. It is likely that these many, diverse activities will be able to elicit a whole range of biological effects, e.g. some stimulatory, some

Figure 16 Proposed sequence of events when REL-C7 cells are exposed to DMSO.



inhibitory. For a particular culture application it is necessary to pre-test batches of serum to determine if they are suitable. Some will be suitable, some will be rejected. This reflects our lack of understanding about the nature of serum and its role in tissue culture. The provision of reliable, chemically-defined, serum-free medium is currently an area of intense investigation by a number of the biotechnology companies.

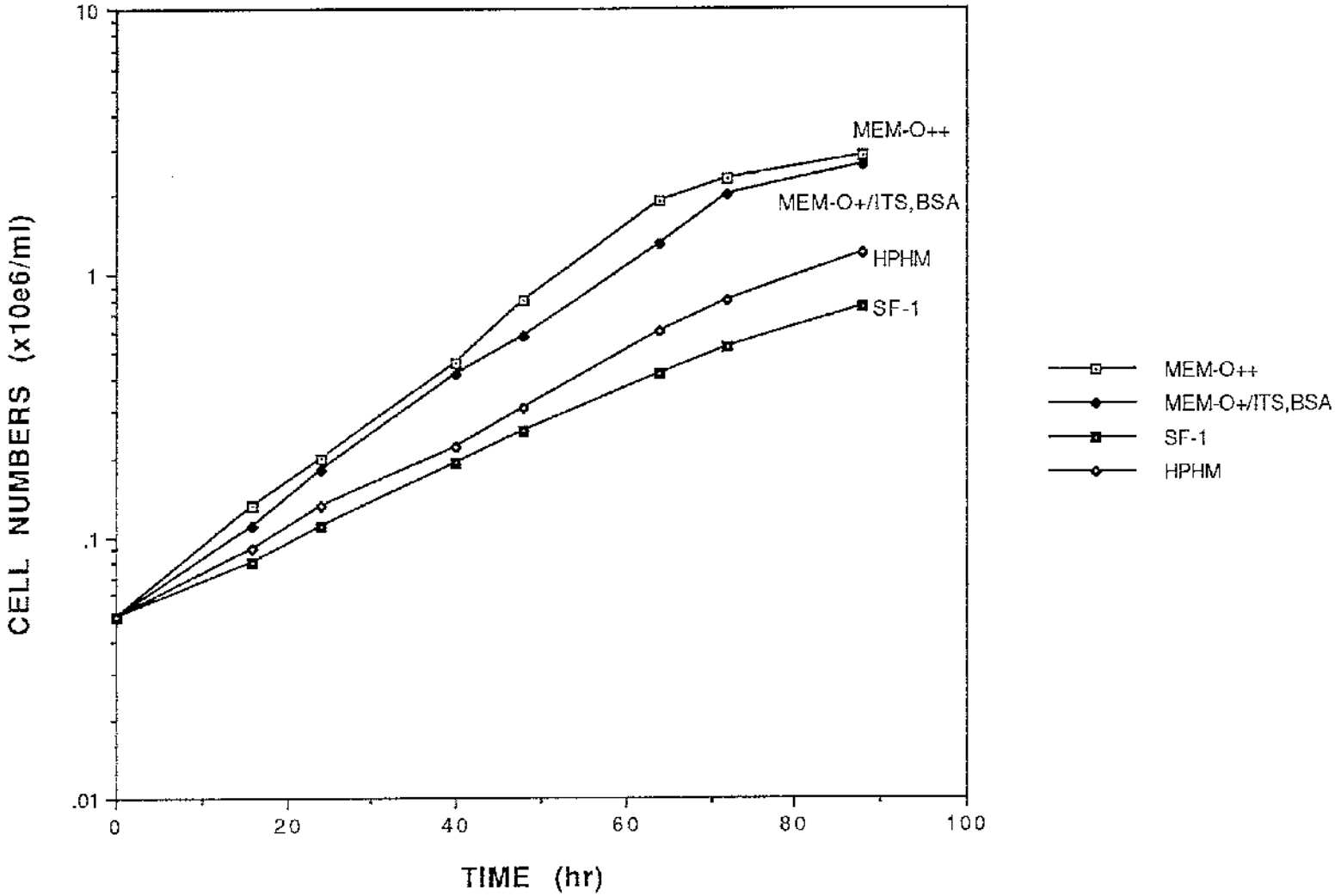
In previous sections, data were presented demonstrating that DMSO can induce REL-C7 cells to differentiate whereby they lose their proliferative ability and follow an erythroid maturation programme. The aim of this part of the work was to identify biological activities that influenced the regulation of differentiation. Since serum contains so many undefined activities, it seemed inappropriate to use serum-supplemented media. The strategy was to prepare a basic culture medium in which all the constituents were chemically defined. By supplementing this medium with a particular growth factor or hormone, it may be possible to see what factors are able to modulate the differentiation process.

Serum-free media were prepared as outlined in Section 2.14. Over a period of time, REL-C7 cells were adapted to these various media. This was done by gradually replacing the serum-containing medium with the appropriate serum-free medium. The cells readily adapted to these media.

To investigate if REL-C7 cells grew equally well in all the different media, growth curves were set up and doubling times calculated (Section 2.15). In addition, the cells were assessed for viability by Trypan Blue exclusion (Section 2.4.1). A representative example is shown in Figure 17.

All 3 serum-free media were, to a greater or lesser extent, sub-optimal. The "in-house" medium, MEM-O±/ITS, BSA (MEM-O± supplemented with Insulin, Transferrin, Selenious Acid and Bovine Serum Albumin) was the best of the three; SF-1 (Northumbria Biologicals) was the worst. MEM-O±/ITS, BSA most closely mirrored MEM-O++. Cells grown in MEM-O++ started to come out of exponential phase after ~ 64 hr of culture and reached a plateau in stationary phase at a cell

Fig.17
Growth curves of REL-C7 cells cultured in various media.



concentration of 2.8 x 10^6/ml. Cells grown in MEM-O±/ITS, BSA lagged behind somewhat, coming out of exponential phase after ~ 72 hr of culture, but nevertheless reaching a plateau at ~ 2.6 x 10^6/ml after ~ 86 hr of culture. The rate of growth in both SF-1 and HPHM started to slow down after ~ 75 hr of culture and neither medium allowed the cell numbers to reach the level achieved with MEM-O++ (2.8 x 10^6/ml). SF-1 cultured cells reached a plateau at ~ 0.8 x 10^6/ml, while HPHM cultured cells reached a plateau at ~1.4 x 10^6/ml. In all the media tested, there was no difference in the cell viabilities, as assessed by Trypan Blue exclusion. In all 4 media, at all the time points shown, high viability was always obtained (≥ 96%) (data not shown). Thus, SF-1 and HPHM media seemed to have no obviously deleterious effect on REL growth. Rather, these media seemed well capable of supporting cell growth, albeit at a sub-optimal level.

Doubling times were calculated (Section 2.15) and data from 6 experiments are summarised in Table 24. There was no significant difference between cells cultured in MEM-O++ and cells cultured in MEM-O±/ITS, BSA ($P>0.1$; student's t-test). There was, however, a significant difference in the doubling times of cells cultured in SF-1 or HPHM compared to MEM-O++ (SF-1, $p<0.001$; HPHM, $p<0.02$).

Thus, of the three serum free media examined, MEM-O±/ITS, BSA seemed superior, and this medium was used for most of the subsequent studies into DMSO - induced differentiation of REL-C7 cells cultured in serum-free conditions.

3.6.1. Differentiation of REL-C7 cells cultured in serum-free conditions.

REL-C7 cells, originally grown in MEM-O++, were adapted to serum-free culture as previous outlined in Section 2.14.

DMSO was used as the potential inducer of differentiation and experiments were set up as described in Sections 2.7 and 2.7.1. The extent of any erythroid differentiation was measured by the benzidine reaction which detects haemoglobin (see Sections 2.8 and 2.8.2).

Table 24

Doubling times of REL-C7 cells cultured in various media.

Results are expressed as mean ± SD of six experiments.

Culture Medium	Doubling Time (hr)	P-value
MEM-O/+	13.7 ± 3.0	-
MEM-O/ ITS, BSA	14.8 ± 1.8	P > 0.1 NS
SF-1	22.0 ± 3.7	P < 0.001
HPHM	18.5 ± 8.6	0.01 < P < 0.02

NS =

not significant

Briefly, tissue culture flasks containing 5 ml of appropriate medium were pre-equilibrated by incubating them at 37°C/5% CO_2/humidified atmosphere for 2-3 hours prior to use. Routinely, cells were set up at a concentration of 0.01 x 10^6/ml. Immediately before inoculating the flasks with cells, DMSO was added to a final concentration of 1.5% (75 µl → 5ml). Obviously, cells adapted to a particular medium were set up in that medium supplemented with DMSO, e.g. cells cultured in SF-1 medium were set up in SF-1 medium/1.5% DMSO. After 4 days of incubation, cells from each flask were aspirated and analysed by the benzidine reaction. Data from 3 experiments are presented in Figure 18.

Only those cells cultured in MEM-O++ (i.e. serum- containing medium) were able to differentiate, reaching a maximum of 49% B+ve. By contrast, all three serum-free media produced <1% B+ve cells (in fact, on the slides examined, any B+ve cells were extremely rare events). This was a quite striking result, and further experiments were set up to try to establish what was required to allow differentiation to proceed.

3.6.2 Serum-supplemented "serum-free medium"

All the differentiation data presented so far have been obtained from REL-C7 cells cultured in MEM-O++/DMSO. In the previous section, the lack of differentiation obtained in the serum-free culture may have been due to the change in basic medium rather than the absence of serum. For example, there may be some inherent property of SF-1 medium that does not allow differentiation to proceed. To exclude this possibility, the three serum-free media were supplemented with FBS to a final concentration of 10%. Once the cells had adapted to these culture conditions, they were exposed to DMSO as described in the previous section. As before, the extent of erythroid differentiation was assessed by the benzidine reaction. Figure 19 shows the results of three experiments.

In contrast to the results from the previous section, all three serum free media, when supplemented with FBS, were capable of supporting differentiation,

Fig.18
Differentiation of REL-C7 cells cultured in serum-free media in the presence of 1.5% DMSO.

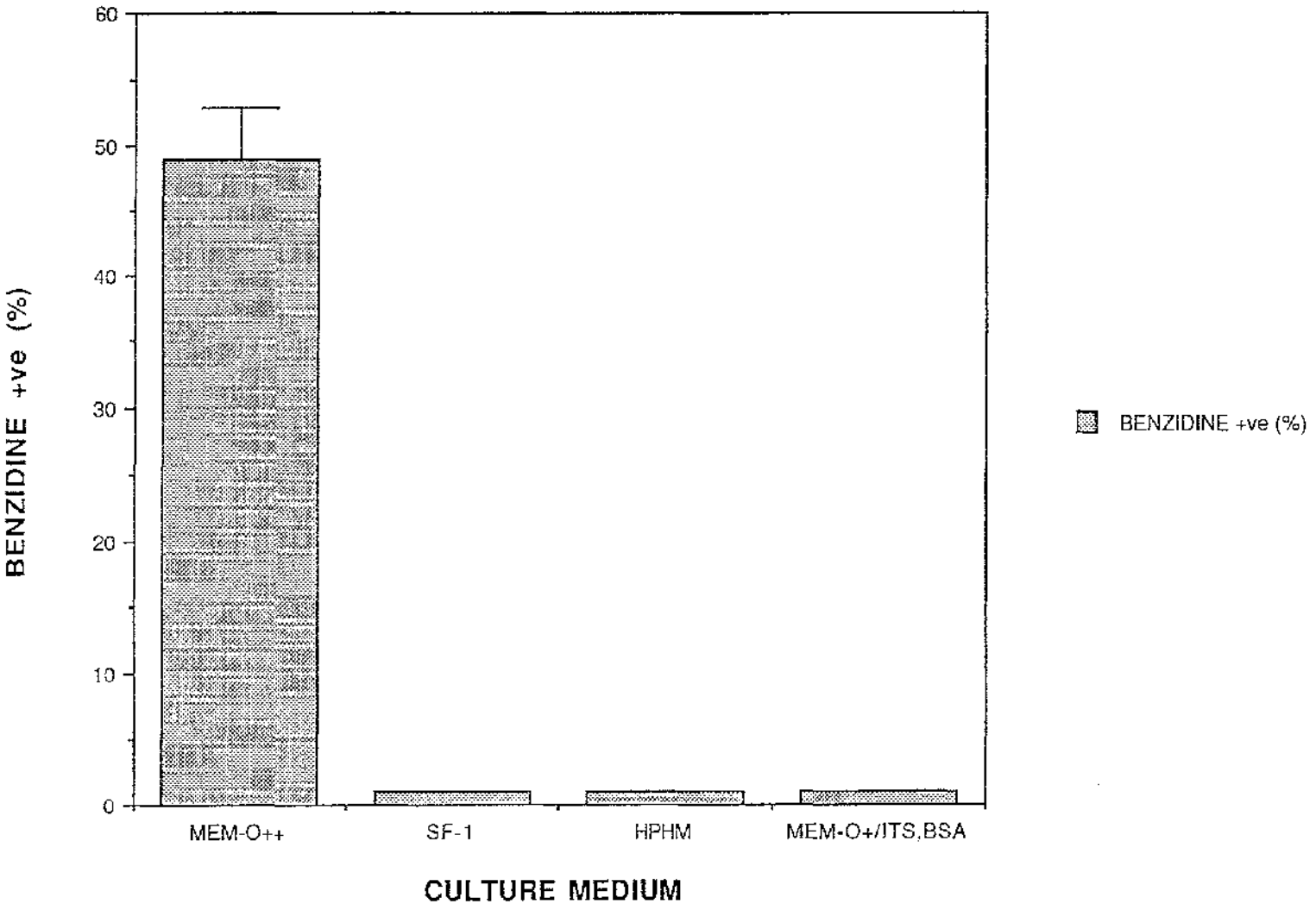
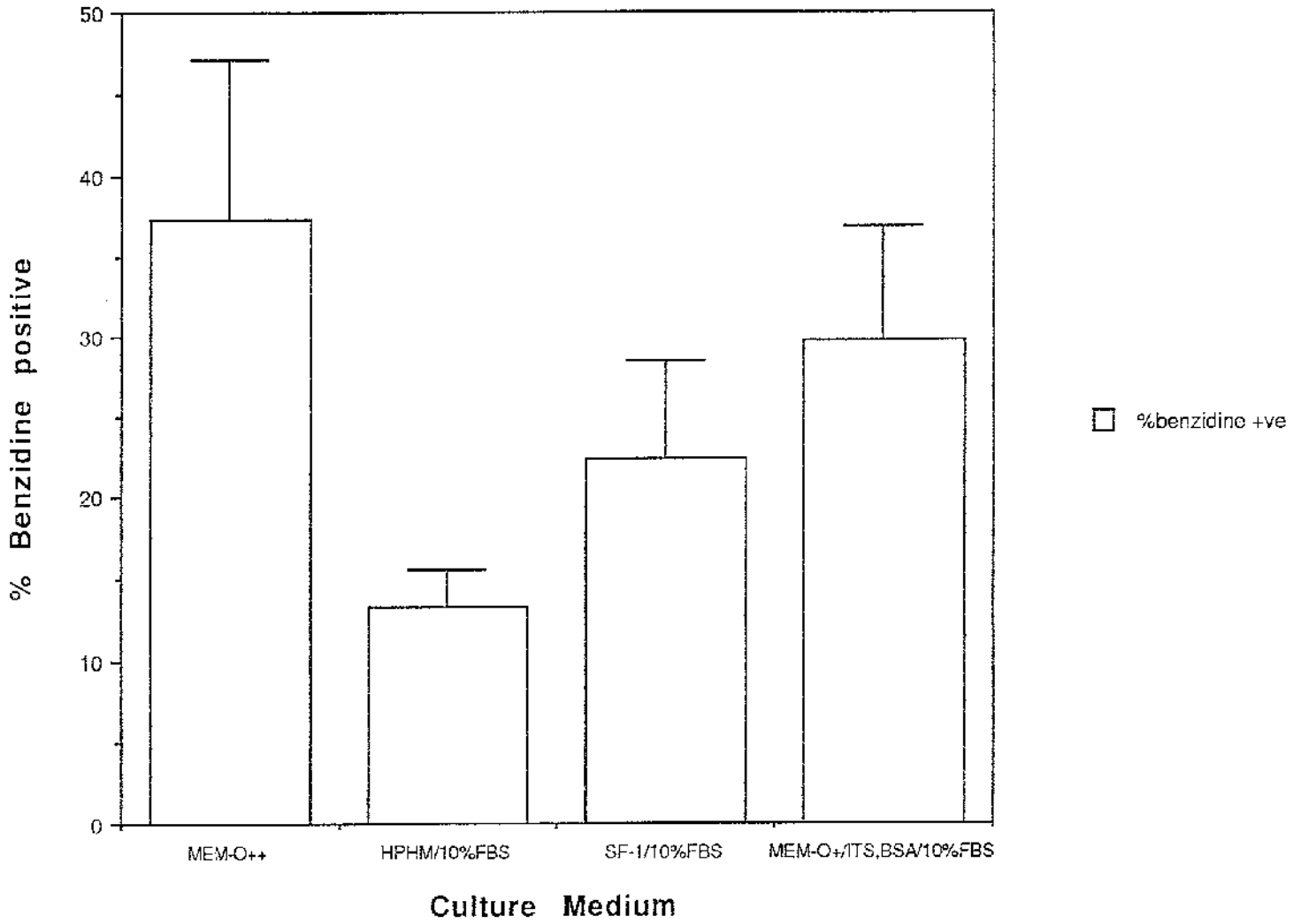


Fig.19
Differentiation of REL-C7 cells cultured in serum-supplemented "serum-free" medium in the presence of 1.5% DMSO



albeit to a reduced extent. Compared to the control (MEM-O++, mean =37% B+ve), all three were inferior:- HPHM/10% FBS, mean =13%; SF-1/10% FBS, mean = 22% B+ve; and MEM-O±/ITS, BSA/10% FBS, mean = 30%. The observation that the serum-free media were inferior is not important. What is important is that, when supplemented with FBS, they were able to sustain an appreciable level of differentiation. This indicates that the basic media do not have any intrinsically adverse effects on differentiation, but rather it is the omission of FBS which is crucial, i.e. no FBS, no differentiation.

3.6.3 Effect of various FBS concentrations on REL-C7 differentiation

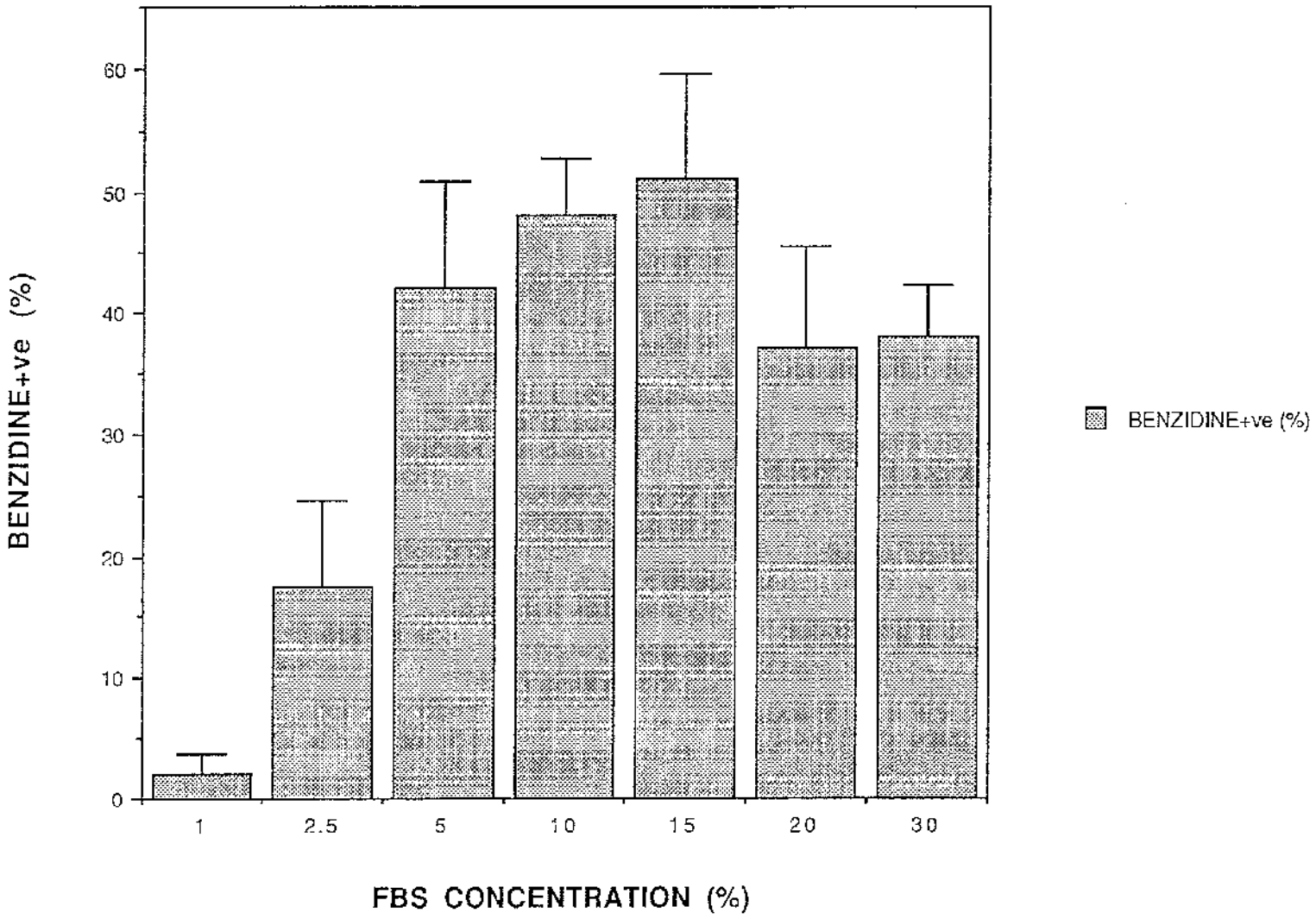
From the previous section, it appeared that FBS, or at least serum of some description, was necessary to sustain DMSO - induced differentiation. Routinely, FBS was used at a concentration of 10%. Experiments were set up to "titre out" this FBS effect, i.e. what concentration of FBS will still sustain significant differentiation?

Several batches of medium were prepared, each supplemented with a different volume of FBS, i.e. MEM-O± with FBS at 30%, 20%, 10%, 5%, 2.5%, 1.0% or 0.5%. REL cells that had routinely been maintained in MEM-O++ (i.e. 10% FBS) were adapted to each of these different FBS concentrations as outlined before (Section 2.16). Following previously described methodologies, these cells were exposed to 1.5% DMSO for 96 hr, and were then assessed for haemoglobin production by the benzidine reaction. Data from 4 experiments are presented in Figure 20.

The optimum range of FBS concentrations giving maximum numbers of B+ve cells was 5-15%. At concentrations higher than these, the proportion of B+ve cells declined, e.g. 30% FBS produced 38% B+ve cells; 20% FBS produced 37% B+ve cells; cf 10% FBS which produced 48% B+ve. Although there appeared to be some variation over the FBS range 5-30%, these differences were not significant (student's t-test).

When the FBS concentration was reduced to 2.5% a marked decline in B+ve cells was detected down to 17.5% (cf 48% B+ve at 10% FBS). This difference was

Fig.20
Effect of various FBS concentrations on differentiation of REL-C7 cells exposed to 1.5% DMSO.



statistically significant ($P<0.01$). When the FBS concentration was further reduced to 1.0%, the proportion of B+ve cells declined again (2% ± 1.8). This drop was significant ($p<0.01$).

Those cultures that had been set up with FBS at 0.5 % were uninterpretable. At this concentration of FBS, REL-C7 cells behaved in a peculiar fashion. Rather than remain in suspension during culture, the cells appeared to become adherent and their gross appearance was altered. This unusual observation was further investigated (see Section 3.7).

In summary, although the optimum concentration of FBS was in the range 5-15%, significant levels of B+ve cells could still be obtained with FBS at 2.5%-30%. Only very low levels (e.g. 2%) of B+ve cells were observed at 1% FBS. Technical limitations prevented FBS concentrations below 1% being investigated. This is in broad agreement with the conclusions in an earlier section (section 3.1.2) in which 1% FBS was the lowest concentration of serum that was still able to support cell proliferation.

3.6.4 "Serum-free" medium supplemented with various sera

In all the previous differentiation studies, FBS has been used as the source of serum, and it has been demonstrated that FBS, at least, is necessary for differentiation to proceed.

Experiments were set up to see if the ability to support differentiation was unique to FBS or whether sera from other sources could substitute for it. Different sera were obtained from a variety of sources (see Section 2.16).Different batches of medium were prepared by supplementing MEM-O± (i.e. no added serum) with one of the following sera:-

(a) mouse,

(b) rat,

(c) rabbit,

(d) horse,

(e) human.

As before, REL cells were adapted to these alternative culture media as described in Section 2.16.

To ensure that REL cells were well maintained in those media containing alternative sera, doubling times were calculated (Table 25). Data from 3 experiments showed that there was no significant difference between any of the sera tested with respect to REL cell proliferation.

These cells that had been adapted to grow in the presence of various sera were now exposed to DMSO to see if they could be induced to differentiate. As before, 5 ml of the appropriate medium were added to a tissue culture flask and allowed to equilibrate for several hours at 37°C/5% CO_2 prior to the addition of 75µl DMSO (i.e. 1.5%) and cells (to a concentration of 0.01 x 10^6/ml). After 4d incubation, the cells were assessed by the benzidine stain for the production of haemoglobin. Data from 3 experiments are presented in Figure 21. Clearly, all 6 of the sera tested were able to sustain REL erythroid differentiation. Furthermore, there were no significant differences in the % B+ve cells between any of the sera. On the evidence presented here, FBS *per se* is not a requirement for differentiation, and in fact, a variety of sera can adequately substitute for FBS.

3.6.5 Heat treatment of FBS: Effects on REL growth and differentiation.

To investigate further the role of serum in REL differentiation, cells were grown in MEM-O± supplemented with heat-treated FBS (see section 2.17). "Heat inactivated" (H.I.) serum was prepared by incubating aliquots of FBS at 56°C for 30 min. "Denatured" (D.N.) serum was prepared by boiling aliquots for FBS for 5 min.

As before, cells were adapted to grow in MEM-O± supplemented with either HI-FBS or DN-FBS. Doubling times were calculated to see what effect, if any, these heat treatments had on REL proliferation (Table 26). HI-FBS was an adequate replacement for FBS. No significant difference was detected in the doubling times between FBS and HI-FBS supplemented MEM-O±. DN-FBS, on the other hand,

Table 25

Doubling times of REL-C7 cells cultured in MEM-O± supplemented with various sera.

Results are expressed as the mean ± SD of three experiments.

Serum Supplement	Doubling Time (hr)	P-value
FBS	14.3 ±1.1	-
Mouse	12.8 ± 0.6	0.5 > P > 0.1
Rat	13.9 ± 1.9	P > 0.5
Rabbit	14.8 ± 0.6	P > 0.5
Horse	14.0 ± 1.4	P > 0.5
Human	14.9 ± 1.4	P > 0.5

Fig.21
Differentiation of REL-C7 cells cultured in MEM-O+ supplemented with various sera in the presence of 1.5% DMSO.

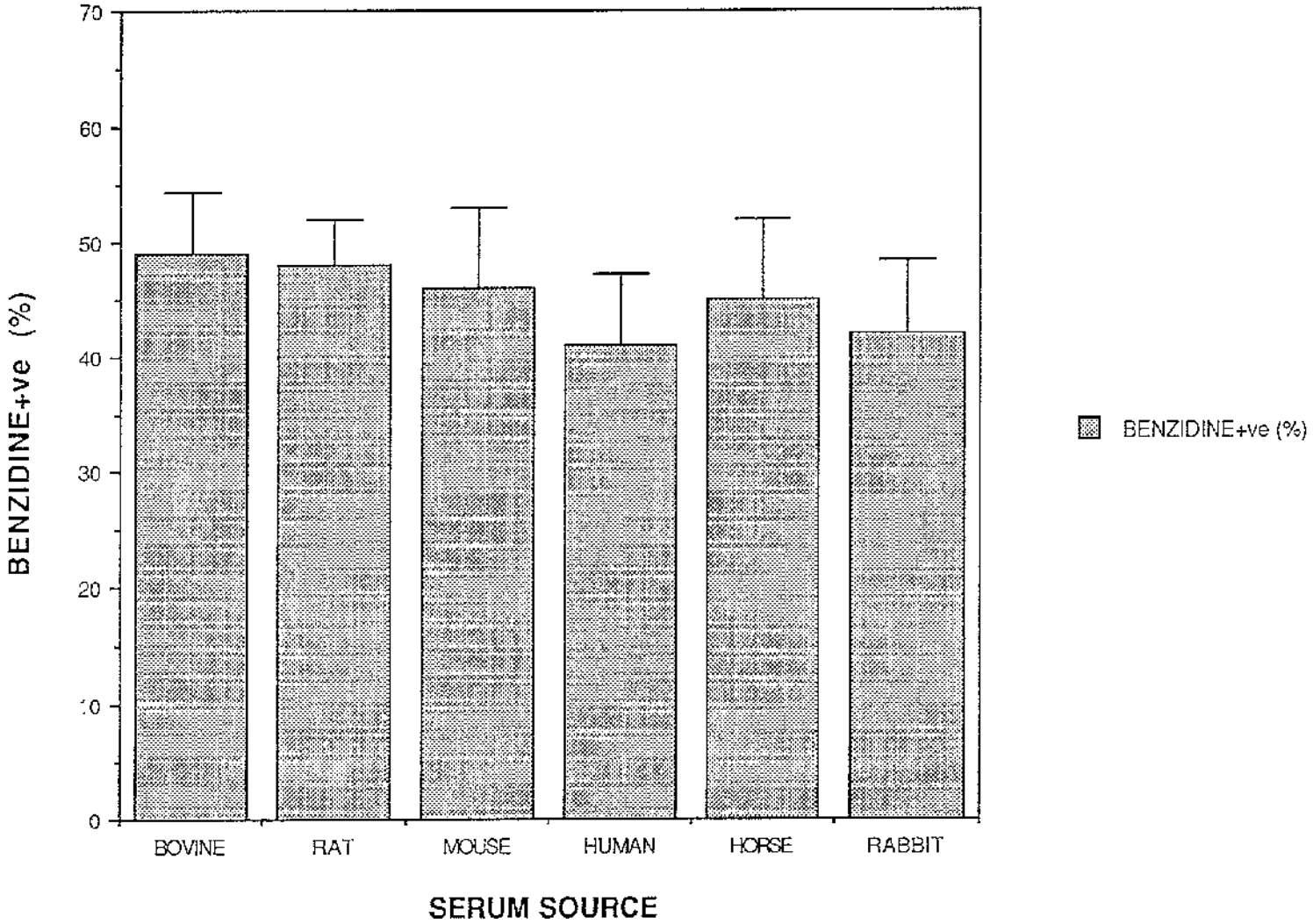


Table 26

Doubling times of REL-C7 cells cultured in MEM-O± supplemented with heat-treated FBS.

Results are expressed as the mean ± SD of three experiments.

Serum Supplement	Doubling Time (hr)	P-value
FBS	13.7 ± 1.2	-
HI-FBS	13.8 ± 1.1	P > 0.5
DN-FBS	21 ± 1.0	P < 0.01

showed a significant extension to the doubling time compared to FBS (21 hr cf 13.7 hr, $p<0.01$, student's t-test).

As before, cells maintained under these culture conditions were exposed to DMSO to induce erythroid differentiation. Data from 3 experiments are presented in Figure 22. Replacing FBS with HI-FBS had no deleterious effect on differentiation. On d.4 in MEM-O±/HI-FBS the percentage of B+ve cells was 53.3 ± 7.1 (mean ± 5.0) compared to 49.7 ± 4.2 obtained in MEM-O++ (i.e. containing FBS). The values were not significantly different ($0.5 > p > 0.1$, student's t-test). By contrast, replacing FBS with DN-FBS prevented any significant differentiation from taking place. In all 3 experiments, the percentage of B+ve cells was always < 1%.

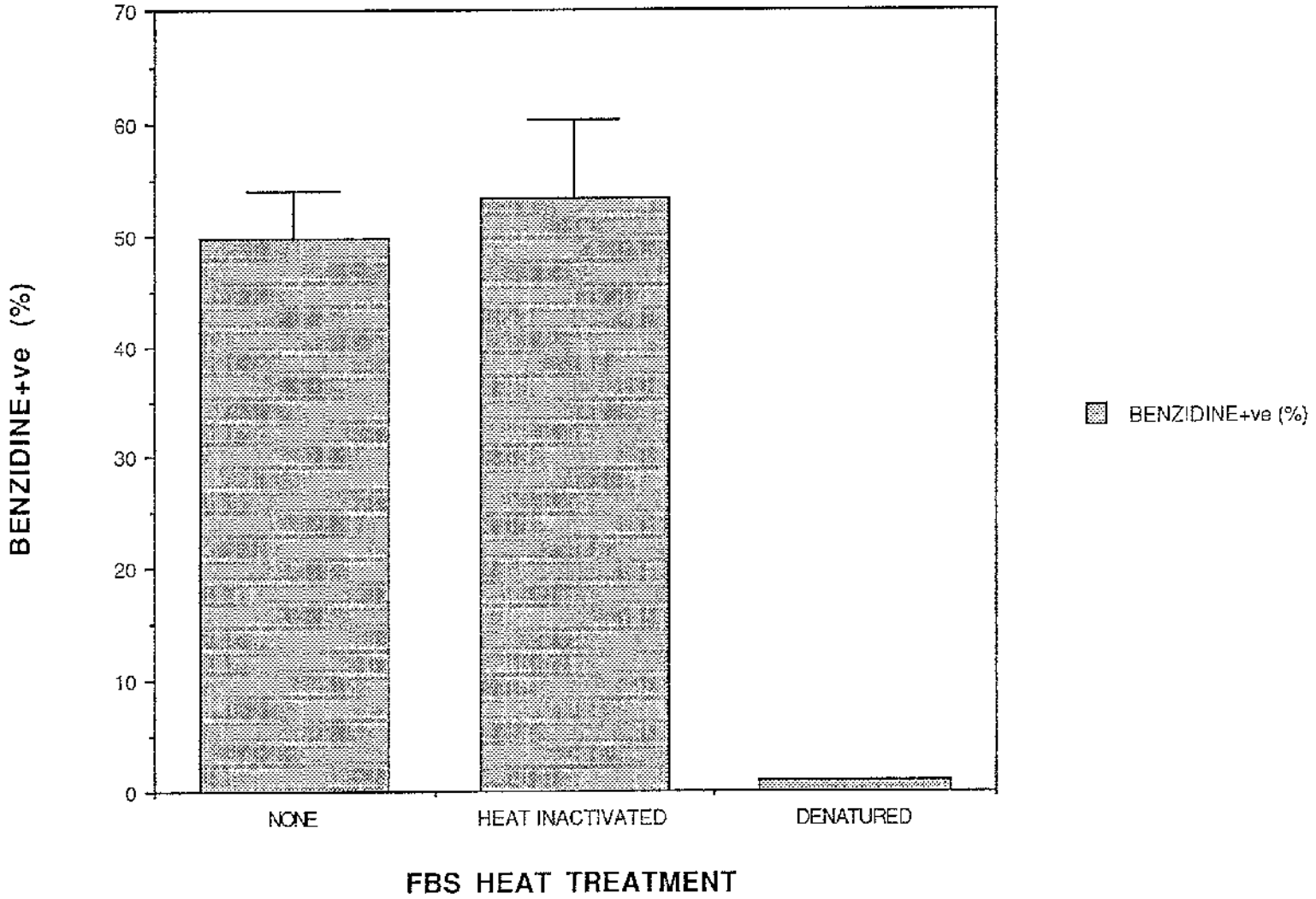
Thus, heat inactivation of FBS affects neither its ability to support REL cell proliferation, nor its ability to allow differentiation to occur. On the other hand, heat denaturation of FBS allows only sub-optimal REL cell proliferation and its ability to support differentiation is completely abrogated. It would appear that some serum factor (s) is required for differentiation to proceed, and that this activity is retained following mild heat treatment. However, following more severe heat treatment, this serum factor (s) loses its activity and differentiation cannot proceed. It is likely that this active serum factor (s) is proteinaceous.

3.6.6 Commitment to differentiation of REL-cells cultured in serum-free medium

Commitment has been defined as the ability to continue differentiation in the absence of inducing agent. It has previously been demonstrated that REL-C7 cells can be committed to differentiate with as little as 8 hr exposure to DMSO, although it requires some 24-48hr to obtain significant commitment.

"Transfer-out" experiments were performed to investigate the role of FBS in commitment. The method used earlier (Section 2.9) was slightly modified. Briefly, REL cells cultured in MEM-O++ were inoculated into a pre-equilibrated tissue culture flask containing 5 ml MEM-O++ with DMSO at 1.5 % (final cell concentration 0.01 x

Fig.22
Differentiation of REL-C7 cells cultured in MEM-O+ supplemented with heat-treated FBS.



10^6/ml). After 24 hr incubation, the culture was poured into a centrifuge tube and the cells centrifuged at 100 g/5 min. The cells were washed twice with MEM-O++ (i.e. no DMSO) and resuspended in 5 ml of fresh, pre-equilibrated MEM-O++ in a second tissue culture flask. These secondary cultures were maintained for a further 72 hr (i.e. 96 hr in total), then the cells were assessed for haemoglobin production by the benzidine reaction.

Similarly, parallel cultures were set up in which REL cells, maintained in MEM-O++, were exposed to DMSO as above. However, on this occasion, after 24 hr of culture, the cells were washed twice with MEM-O± /ITS, BSA to remove any residual DMSO, and also to remove/reduce any FBS. These cells were established in secondary cultures in MEMO-±/ITS, BSA (ie no DMSO and no FBS), and, after a further 72 hr, they were assessed for B+ve cells.

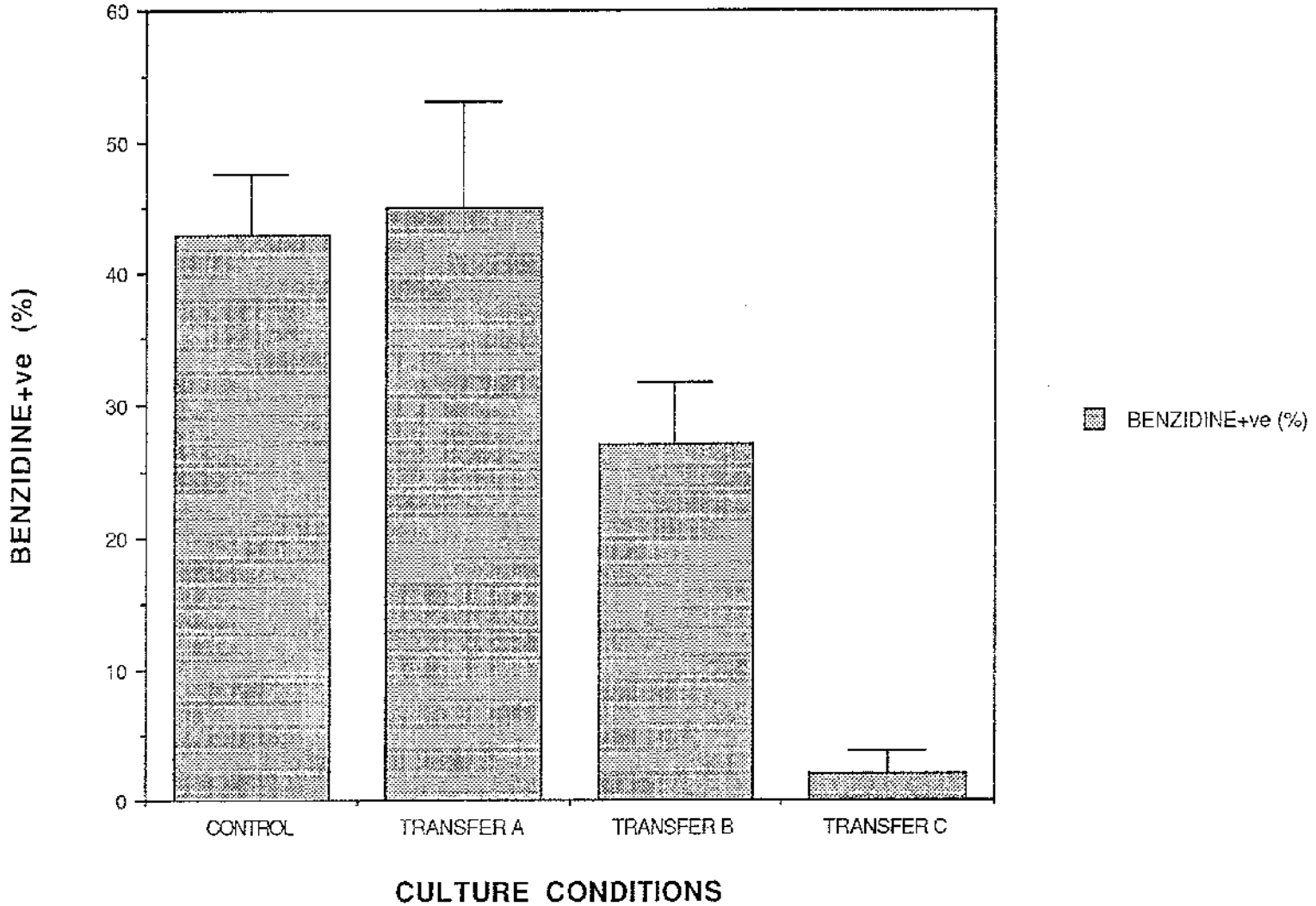
Lastly, further cultures were set up in which REL cells that had been maintained in MEM-O±/ITS, BSA (no FBS) were inoculated into a fresh flask of 5 ml of MEM-O±/ITS, BSA with 1.5% DMSO. After 24 hr incubation, the cells were washed free of any contaminating DMSO as before and re-established in a secondary culture flask containing MEM-O++ (i.e. serum-containing). After a further 72 hr incubation, the cells were assessed for B+ve cells.

In Summary,

	Cell cultured for 24 hr in	*transferred into*	Cells cultured for 72 hr in
A.	MEM-O++/DMSO	→ → → → → → → → →	MEM-O++
B.	MEM-O++/DMSO	→ → → → → → → → →	MEM-O±/ITS, BSA
C.	MEM-O±/ITS, BSA/DMSO	→ → → → → →	MEM-O++

The data from 3 experiments are summarised in Figure 23. A control culture was also set up in which REL cells were maintained in MEM-O++ supplemented with 1.5% DMSO, uninterrupted for 96 hr.

Fig.23
Effect of serum-free medium on the commitment of REL-C7 cells exposed to 1.5% DMSO for 24hr.



In the control culture, 43% (43 ± 4.6, mean ± SD) of the cells were B+ve. In test flask A (i.e. cells exposed to DMSO in the presence of FBS, then transferred to FBS- containing MEM-O), 45% (45 ± 6.1) of the cells were B+ve. This demonstrated that there was a high level of commitment to differentiate after 24 hr exposure to DMSO. Although the test value of 45% B+ve was slightly higher than the control value of 43%, this difference was not significant ($p>0.5$, student's test).

Interestingly, in test flask B (i.e. cells exposed to DMSO in the presence of FBS, then transferred to serum-free MEM-O), 27% (27 ± 4.6) of the cells were B+ve. Although this value of 27% is significantly lower than the control value of 43% ($0.02 > P>0.01$, student's t-test), it still represents an appreciable amount of differentiation. Previously it was shown (Sections 3.6.1, 3.6.2) that serum, of some description, was necessary for differentiation to proceed. Here it has been demonstrated that once REL cells have been committed to differentiate, then, to some extent at least, serum is no longer required for differentiation. Thus, after 24 hr exposure to DMSO in MEM-O++, many cells have already "made the decision" to differentiate and subsequent removal of serum does not affect the differentiation process.

The last combination that was set up (flask C), looked at cells that were exposed to DMSO in serum-free medium, and were then transferred to serum-containing medium. Only very low, barely detectable, levels of B+ve cells were obtained, 2% ± 1.7. Obviously, this figure is highly significantly different from the control, and indeed from both of the other test cultures. It would appear that very few cells became committed to differentiation when exposed to DMSO in the absence of serum. In addition, these data indicate that serum may not be required to support the entire differentiation process, but rather it is necessary only in the critical stages of differentiation when cells became irreversibly committed to differentiate.

It is possible that the inability of REL cells to differentiate in the absence of serum may be due to some profound phenotypic change induced in the cells by serum-deprivation, i.e. they may have lost the capacity to differentiate. To test this possibility, cells that had been adapted to MEM-O±/ITS, BSA (i.e. serum-free) were

"re-adapted" back to MEM-O++ (i.e. serum-containing). Subsequently, when these cells were exposed to DMSO, a significant, albeit sub-optimal, level of B+ve cells was obtained (Figure 24), 19% ± 6.2 (cf control 43 ± 4.6). This demonstrated that prolonged maintenance in serum-deprived conditions did not abrogate the ability of cells to differentiate. Cells inherently retained this ability to differentiate and were able to express this under suitable culture conditions.

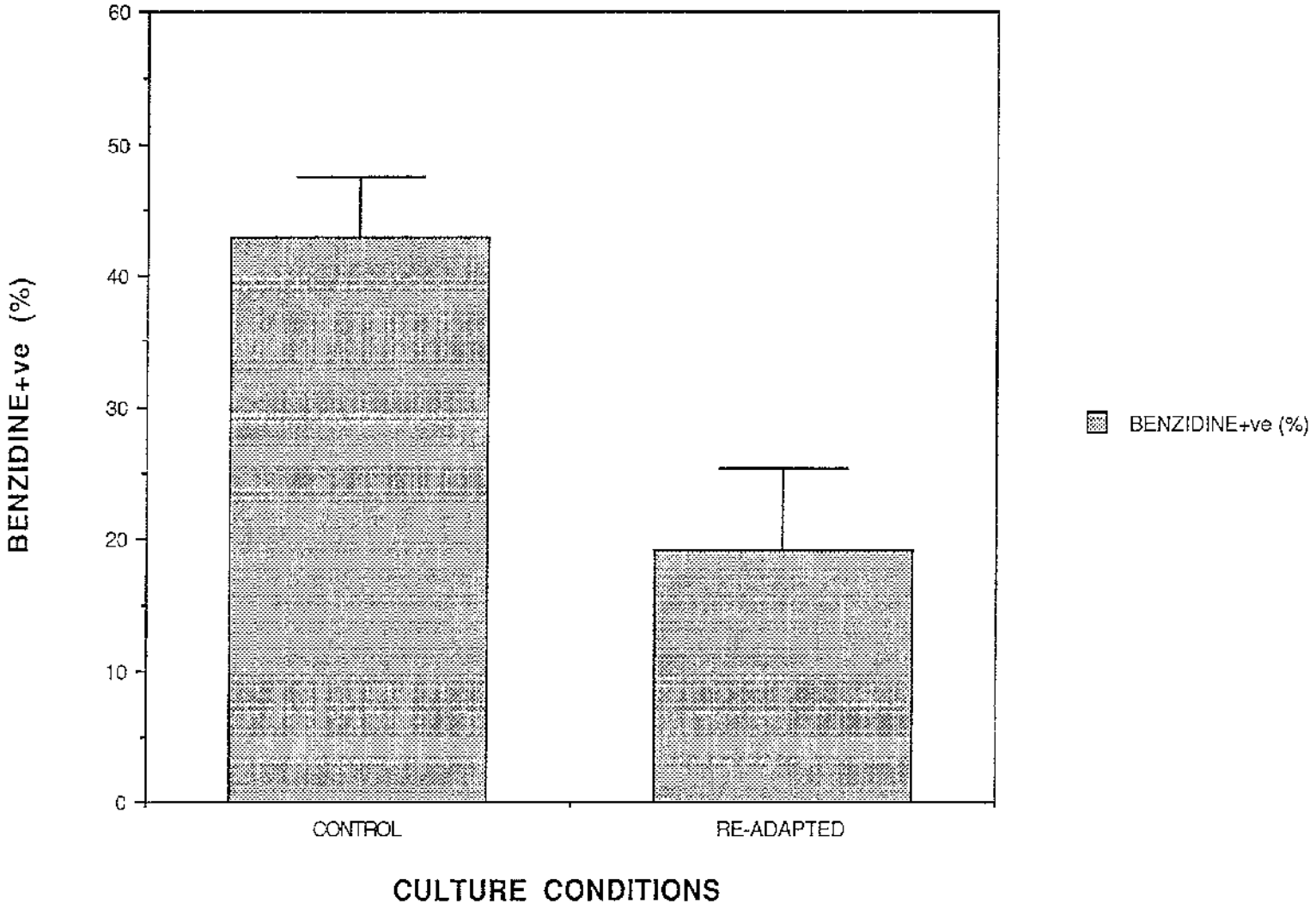
3.6.7. Addition of recombinant growth factors to serum-free culture. Effect on DMSO- induced differentiation

It has been clearly demonstrated that serum contains some activity(s) which is required to enable cells, in the presence of an inducer, to enter a differentiative phase in which they increasingly lose the capacity to proliferate and instead acquire a much more mature phenotype as demonstrated by morphology and the production of haemoglobin. More precisely, this activity seems to be necessary for the "decision-making" process, i.e. commitment to differentiate. The required factor appears to be a common serum component whose activity is not affected by heat-inactivation, but whose activity is destroyed by heat denaturation. In all likelihood, the factor is proteinaceous in nature. What, then, is this activity?

The manner in which low molecular weight inducers (like DMSO) exert their effects has yet to be resolved. Similarly, the culture conditions that influence differentiation are ill-defined. A major impediment to this has been the necessary inclusion of serum in cell culture. The myriad activities present in serum make it impossible to delineate the factors that influence differentiation. Clearly, the use of a serum-free medium, in which all the constituents are defined, is an attractive proposition.

One strategy used to develop chemically-defined media is the so-called "unifying approach" (174). In effect, this involves designing a basic culture

Fig.24
Differentiation of REL-C7 cells re-adapted to serum-containing conditions (MEM-O++) from serum-free conditions (MEM-O+/ITS,BSA)



medium in which all the constituents are chemically-defined. By supplementing this basic medium with a particular hormone or growth factor (or combination of factors), it should be possible to see what influence, if any, they have on, say, differentiation.

A range of cytokines/growth factors (GF) was assessed for any effect on DMSO-induced differentiation: -

(a) Interleukin-6 (IL-6),

(b) Interleukin-11 (IL-11),

(c) Granulocyte colony stimulating factor (G-CSF),

(d) Macrophage colony stimulating factor (M-CSF),

(e) Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF),

(f) Kit Ligand (KLS),

(g) Macrophage Inflammatory Protein- 1α (MIP-1α).

(see Section 2.18)

Stock MEM-O±/ITS, BSA was prepared. Aliquots of 20 mls were dispensed into Universal containers. Each cytokine/GF was studied at 3 different concentrations. Thus, to each aliquot of 20 ml was added one concentration of one cytokine/GF. The concentration ranges were:-

(a)	IL-6	:	2, 20, 200 ng/ml.
(b)	IL-11	:	1, 10, 100 ng/ml.
(c)	G-CSF	:	1, 10, 50 µl/ml
(d)	M-CSF	:	1, 10, 100 ng/ml
(e)	GM-CSF	:	0.5, 5, 50 U/ml
(f)	KLS	:	2, 20, 50 µl/ml
(g)	MIP-1α	:	20, 200, 500ng/ml.

Initial studies assessed the proliferative status of cells grown in the presence of a particular cytokine. Briefly, cultures were set up in 24-well Tissue culture plates

(Costar). The 16 wells around the perimeter of the plate received 2ml sterile distilled water. The central 8 wells were used to establish cultures (Filling the outer wells with water minimises evaporation and reduces the tendency of the medium to become hypertonic). REL-C7 cells that had been adapted to MEM-O±/ITS, BSA were used to inoculate the well-cultures. Cells were set up at 1 x 10^4/ml in a 2 ml volume. Doubling times were calculated as before (Section 2.15). Data from 2 experiments are shown in Table 27.

These data indicated that none of the cytokines/GF's appeared to have any great effect on REL-C7 proliferation, i.e. neither stimulatory nor inhibitory. All cells seemed to grow perfectly well, no matter what cytokine was added. For convenience, therefore, one concentration of each cytokine was selected for use in subsequent differentiation studies. The "middle" concentration (i.e. the recommended concentration) was chosen for each cytokine.

REL-C7 cells were set up at 1 x 10^4/ml in tissue culture flasks in 5 ml of MEM-O±/ITS, BSA supplemented with a single cytokine and DMSO at 1.5%. After 4 days the cells were assessed for haemoglobinazation (see Table 28).

As demonstrated before, MEM-O±/ITS, BSA was unable to support DMSO-induced differentiation ("No addition" in Table 28). Equally, when MEM-O±/ITS, BSA was supplemented with a single cytokine at the concentration indicated, no significant haemoglobinzation was detected. A few B+ve cells were observed but the numbers were small, not greater than the background level of spontaneous differentiation described in Section 3.5.2. As a positive control, a culture of REL-C7 cells was set up in MEM-O++ with 1.5% DMSO. The subsequent level of haemoglobinization was satisfactory (38%± 5.6, mean ± SD).

As a further investigation, some cytokine combinations were studied. Most of the GF's available are regarded as somewhat restricted in the lineages that they can influence. However, KLS (SCF) and MIP-1α exert at least some of their effects on more primitive cells, often in synergy with other cytokines. The first part of the study combined KLS with each of the other cytokines listed. The second part of the study

Table 27

MEM-O± / ITS, BSA supplemented with various cytokines. Effect on REL-C7 doubling time.

Cytokine / Growth Factor	Doubling Time (hr) Experiment 1	Doubling Time (hr) Experiment 2
No addition	15.2	16.0
IL-6 2 ng/ml	16.1	15.7
20	14.8	14.8
200	14.2	16.0
IL-11 1 ng/ml	15.0	13.5
10	15.9	15.3
100	16.7	16.4
G-CSF 1 µl/ml	16.6	17.8
10	16.6	15.6
50	15.0	15.5
M-CSF 1 ng/ml	17.0	18.1
10	16.0	17.7
100	14.9	14.6
GM-CSF 0.5 U/ml	18.6	16.4
5.0	16.2	13.5
50	16.7	15.7
KLS 2 µl/ml	14.9	15.8
20	14.3	18.0
50	15.4	16.5
MIP-1α 20 ng/ml	14.9	12.7
200	15.2	13.9
500	15.0	16.7

Table 28

DMSO-induced differentiation of REL-C7 cells cultured in MEM-O± / ITS, BSA supplemented with various cytokines.

Results are expressed as the mean ± SD of three experiments

Cytokine Addition	% B+ve
No addition	< 1
IL-6 (20ng/ml)	< 1
IL-11 (10ng/ml)	< 1
G-CSF (10µl/ml)	< 1
M-CSF (10ng/ml)	< 1
GM-CSF (5U/ml)	< 1
KLS (20µl/ml)	< 1
MIP-1α (200ng/ml)	< 1
MEM-O++ Control	38 ± 5.6

combined MIP-1α with each of the other cytokines. Again, REL-C7 cells were set up at $1x10^4$/ml in tissue culture flasks in a 5 ml volume with DMSO at 1.5%. The proportion of cells staining B+ve was assessed as before. Data from 3 experiments are shown in Table 29. Clearly, the cytokine combinations listed were insufficient to support DMSO-induced differentiation. In fact, with all the cytokines used, the lack of effect was quite spectacular! No significant differentiation at all was detected. Consequently, no further cytokines or cytokine combinations were investigated, and the identity of the serum factor which supported differentiation remained unresolved. Considering the many and varied activities present in serum, this section of the work was something of a "fishing" exercise. Unfortunately, no fish were caught!

3.7 Phenotypic alterations in cultured REL-C7 cells

In a previous section (Section 3.6.2) it was shown that omission of FBS from the culture medium prevented cells from differentiating. To try to determine the lowest concentration of FBS that would still support differentiation, cultures were set up in which MEM-O± was supplemented with a range of FBS concentrations from 0.5 % - 30% (Section 3.6.3). An unexpected observation was that cells cultured in 0.5% FBS underwent a profound alteration in their gross morphology and in their growth characteristics.

3.7.1. Gross morphological changes

As outlined in Section 3.6.3, attempts were made to adapt REL-C7 cells to grow in culture medium supplemented with a range of FBS concentrations. As part of this investigation, cells that had previously been cultured in MEM-O± /FBS 1% were seeded into flasks containing MEM-O± /FBS 0.5%. Within 24 hr of initiation of these cultures, significant numbers of cells had adhered to the plastic surface of the tissue culture flask. At this stage, many cells were still in free suspension. Over the next few days of culture, the proportion of cells that were adherent increased

Table 29

DMSO-induced differentiation of REL-C7 cells cultured in MEM-O± / ITS, BSA supplemented with cytokine combinations.

Results are expressed as the mean ± SD of three experiments.

Cytokine Combination	% B+ve
No Addition	< 1
KLS + IL-6	< 1
+ IL-11	< 1
+ G-CSF	< 1
+ M-CSF	< 1
+ GM-CSF	< 1
+ MIP-1α	< 1
MIP-1α + IL-6	< 1
+ IL-11	< 1
+ G-CSF	< 1
+ M-CSF	< 1
+ GM-CSF	< 1
MEM-O+− Control	44 ± 6.4

progressively until by about day 4 of culture, relatively few cells remained in suspension.

This adherent interaction with the plastic surface was very strong. Gentle, lateral rocking of the culture flasks failed to dislodge appreciable numbers of adherent cells. Even more vigorous physical agitation of the flasks left many cells still attached to the surface. Effective release of the cells from the plastic could, however, be achieved by drawing up some culture medium into a Pasteur pipette, and then forcibly expelling the liquid, in a fine jet, directly at the adherent cells. This resulted in the vast majority of cells being released into the supernatant. Interestingly, if such cultures were returned to the incubator (or fed and returned to the incubator) by the following day most of the cells had re-adhered.

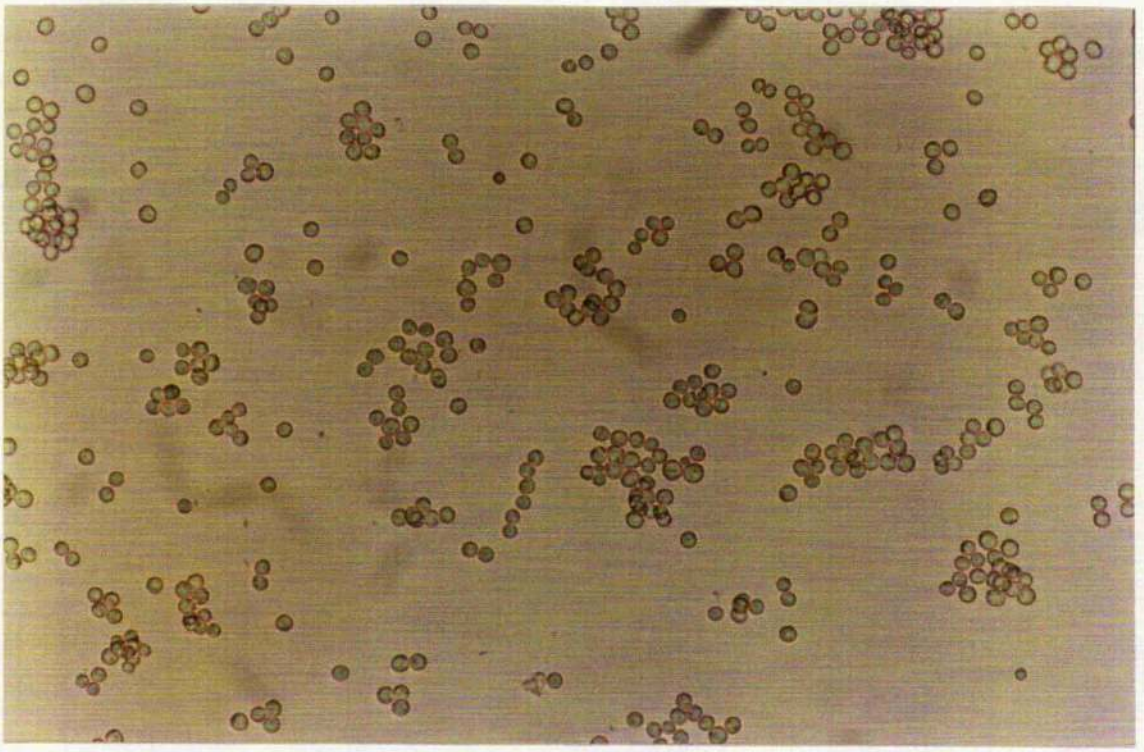
It appeared that this plastic adherence was a response to a reduced FBS concentration. This was not strictly true. An additional batch of medium was prepared in which MEM-O± /FBS 0.5% was supplemented with BSA at a final concentration of 1% (see Section 2.14). When REL cells were inoculated into this medium, the vast majority remained in suspension, although some cells, over a period of days, did show a tendency to adhere. These observations indicated that the adherent phenomenon was more likely due to reduction in protein concentration below a critical level, rather than a reduction in FBS *per se*. This is corroborated by the fact that REL cells could be maintained in suspension for weeks in medium that did not contain FBS, but did contain BSA (Section 3.6).

In addition to this shift from suspension culture to the formation of an adherent monolayer, the appearance of individual cells was also profoundly altered. Cells maintained in suspension culture appeared spheroid and light refractile with a smooth cell surface (Plate 6A). In contrast, adherent cells appeared "flattened" onto the plastic surface and were of irregular shape. Many cells had cytoplasmic extensions radiating out from the cell in all directions (pseudopodia) (Plate 6B). These cells were not dissimilar in appearance to peripheral blood, plastic-adherent, mononuclear cells i.e. monocyte/macrophage cells.

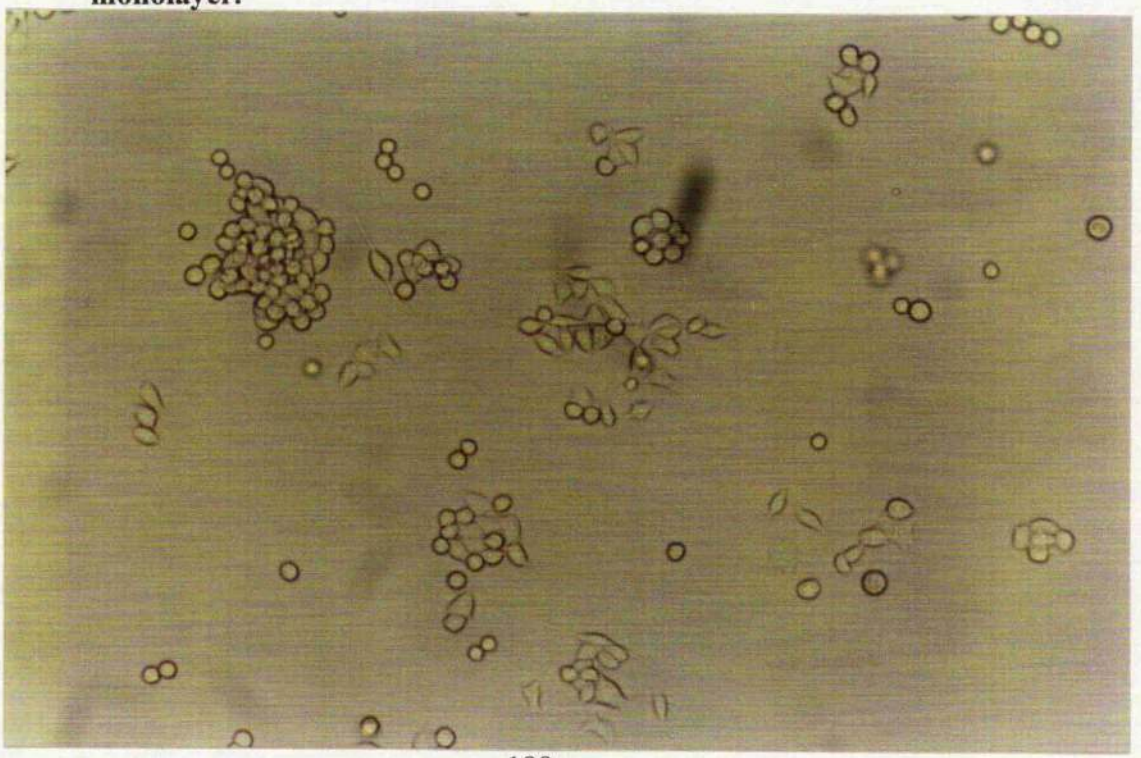
Plate 6

Magnification 400X.

A. Gross morphological appearance of REL-C7 cells grown in suspension culture.



B. Gross morphological appearance of REL-C7 cells grown as an adherent monolayer.



3.7.2. Staining of adherent REL cells

Morphological analyses also showed profound alterations in adherent REL-C7 cells compared to the usual suspension culture cells. Adherent cells were released from the plastic surface by vigourous pipetting and cytospin preparations were made (Section 2.19.4). The slides were allowed to air-dry and were then stained in Wright's stain. From Plate 7, it is obvious that these cells appear substantially different from REL-C7 cells maintained in suspension culture (in MEM-O++) (cf. Plate 4A), and from REL-C7 cells that have differentiated along an erythroid pathway (cf. Plate 4B). These adherent cells have the characteristic appearance of macrophages.

A range of specialised staining techniques was set up on these adherent cells in an attempt to characterise them (Section 2.19) :-

(a) Periodic acid-Schiff (PAS). This stain can be useful in identifying erythroid cells. Often, erythroblasts show heavy PAS positivity.

(b) Sudan Black B. A positive Sudan reaction is associated with myeloid cells, i.e. of the granulocyte and monocytic series.

(c) Alpha Naphthyl Acetate Esterase (α-NAE). This can be useful in identifying cells of the monocyte/macrophage series.

The results are presented in Table 30. Suspension culture REL-C7 cells were singularly unreactive with any of the stains that were set up. Although REL-C7 cells from adherent cultures were negative for PAS and Sudan Black, they were strongly positive with α-NAE (Plate 8), as indicated by the prominent black granulation.This positive result suggested a monocyte / macrophage lineage.

3.7.3. Immunophenotypic analyses by flow cytometry

From the gross morphological appearance of the cells and the pattern of staining with α-naphthyl acetate esterase, it seemed that these REL cells had begun to express characteristics associated with myeloid (monocyte/macrophage) differentiation. In an attempt to corroborate this, REL cells were stained with a panel of monoclonal antibodies :-

Antibody	Specificity
OX-1	rat leucocyte common antigen (LCA)
OX-4	rat Ia (immune associated)
OX-41	rat macrophage
OX-44	all rat myeloid, and peripheral lymphocytes
OX-45	rat surface glycoprotein on haematopoietic cells, and endothelial cells
ED-1	rat monocyte / macrophage

The choice of antibodies was somewhat arbitrary, the main limitation being the range of anti-rat antibodies which was available.

Prior to immunphenotypic analyses, adherent REL-C7 cells were recovered from tissue culture flasks by vigourous pipetting. For comparison, REL-C7 cells from suspension cultures were used, i.e. maintained in MEM-O[illegible] under standard conditions. In addition, normal rat bone marrow cells were also analyzed to give an idea of the normal pattern of expression obtained from fresh haematopoietic material rather than established cell lines (the bone marrow cells were obtained by flushing out the contents of a rat femur).The staining procedure is outlined in Section 2.20. After staining, the percentage of cells expressing a particular antigen was determined by flow cytometry (FACScan, Becton Dickinson). Data from 2 experiments are shown in Table 31.

Virtually all normal rat bone marrow cells were positive for OX-1 and OX-45. In addition, the antigens associated with OX-41 and OX-44 were expressed on the majority of bone marrow cells (82% and 86% respectively). Only low levels of staining were obtained with OX-4 and ED-1 (17% and 15% repectively).

Plate 7

Morphology of REL-C7 cells from adherent monolayer cultures by Wright's stain.

Magnification 1000X.

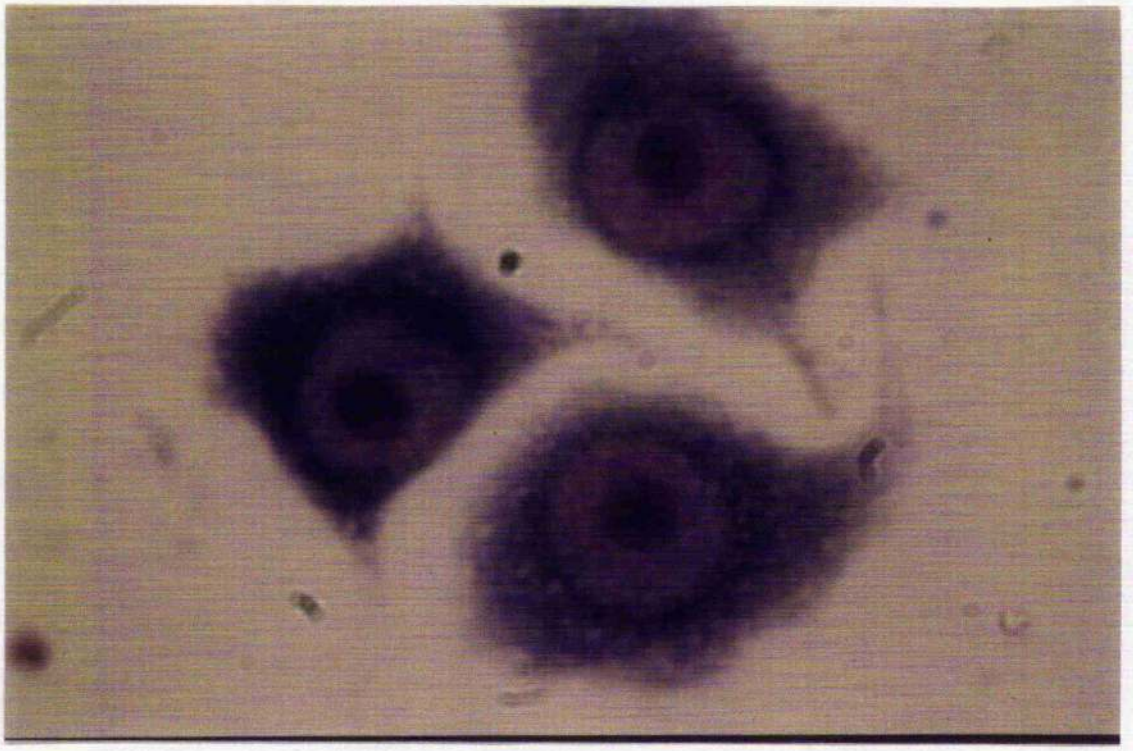


Table 30

Cytochemical analyses of REL-C7 cells grown in suspension culture or in adherent monolayers.

Stain	Suspension Culture	Adherent Culture
PAS	-	-
Sudan Black	-	-
α-NAE	-	++++

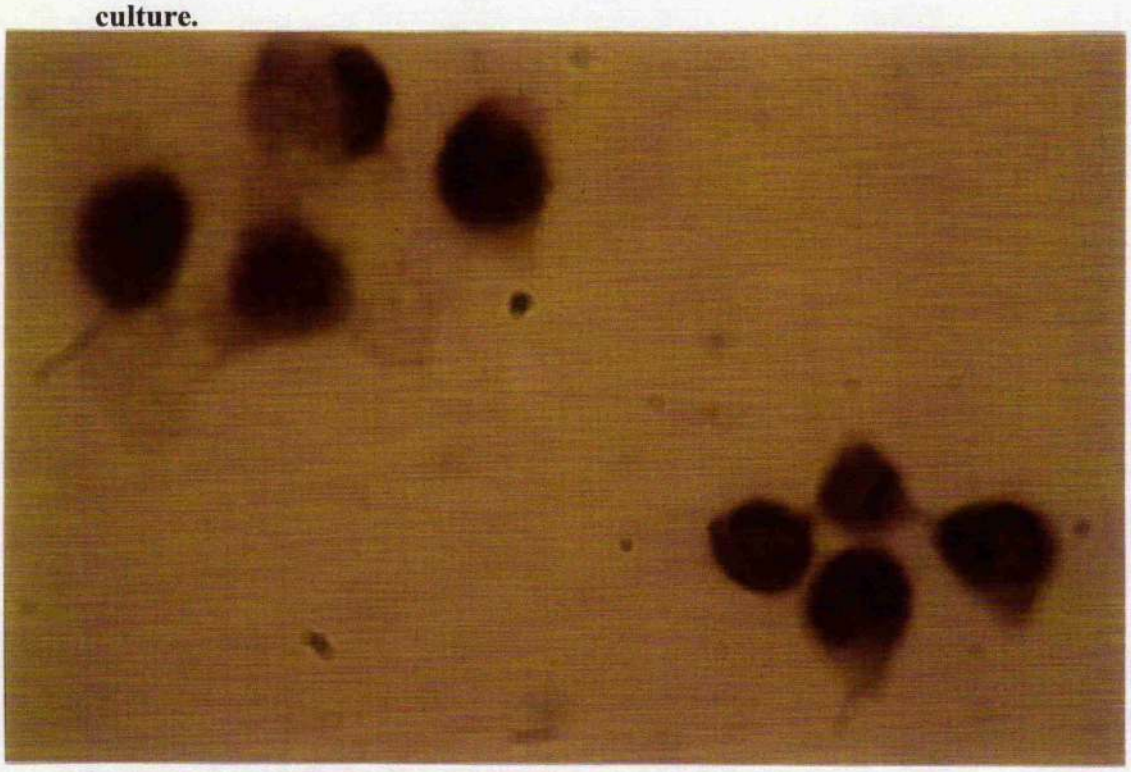
- = negative,

++++ = strongly positive.

Plate 8

Magnification 400X.

A. α-naphthyl acetate esterase staining of REL-C7 cells grown in suspension culture.



B. α-naphthyl acetate esterase staining of REL-C7 cells grown as an adherent monolayer.

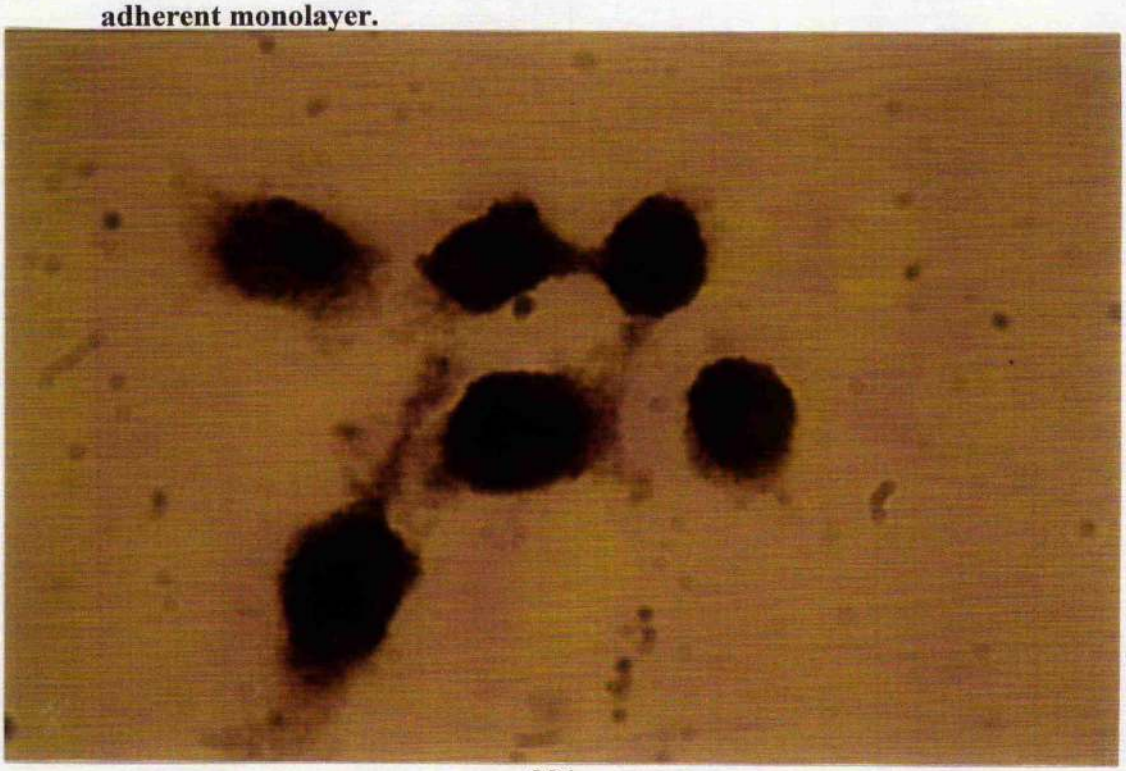


Table 31

Immunophenotypic analyses of various cell populations.

Results are from two experiments.

Values are the means of the percentages of cells positive for that particular antibody.

Monoclonal Antibody	Normal Rat Bone Marrow Cells	REL-C7 Suspension Culture	REL-C7 Adherent Monolayer
OX-1	>95	35	38
OX-4	17	<5	<5
OX-41	82	<5	63
OX-44	86	<5	<5
OX-45	>95	78	70
ED-1	15	<5	<5

For the characterisation of REL-C7 cells, some of the antibodies were not particularly informative. For example, antibodies OX-4, OX-44, and ED-1 were negative (<5% positive) for both suspension cells and adherent cells. With two other antibodies, some degree of positivity was obtained. The antigen identified by OX-1 was expressed on 35% of suspension cells and 38% of adherent cells. The OX-45 antigen was expressed at a higher level, with 78% of suspension cells and 70% of adherent cells being positive. The important point here is, not so much the degree of expression of the antigens, but rather the fact that there was no difference in expression between suspension cells and adherent cells.

OX-41, on the other hand, showed a significant difference. Suspension cells were negative (<5% positive) for OX-41. Adherent cells were strongly positive (63%) for expression of the antigen associated with OX-41. Since OX-41 identifies a macrophage marker, this observation provided further evidence that these adherent REL-C7 cells had developed myeloid (i.e. non-erythroid) characteristics.

3.7.4 Scanning Electron Microscopy (SEM) of adherent REL-C7 cells

It was previously demonstrated (Section 3.5.7) that profound alterations to the cell surface occurred when REL-C7 cells were induced to differentiate along an erythroid pathway following exposure to DMSO. The preceeding sections have indicated that maintenance of REL-C7 cells in low protein culture conditions induced the cells to mature along a myeloid pathway, or more precisely, a monocyte / macrophage pathway.

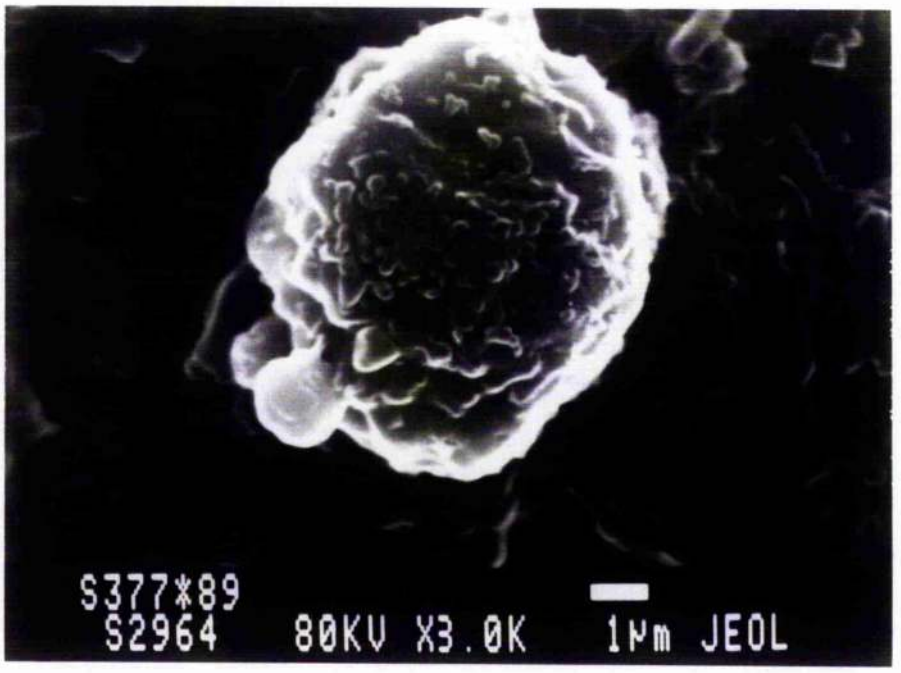
Following the same procedure as before (Section 2.13), REL-C7 cells that had become adherent following maintenance in low protein culture medium (MEM-O± / FBS 0.5%) were prepared for scanning electron microscopy. Striking differences were seen between suspension cells and adherent cells (Plate 9).

As before (Section 3.5.7), cells maintained in suspension culture appeared spheroid with a cell diameter of around 10-12μm. The surface showed many protrusions and ridges.

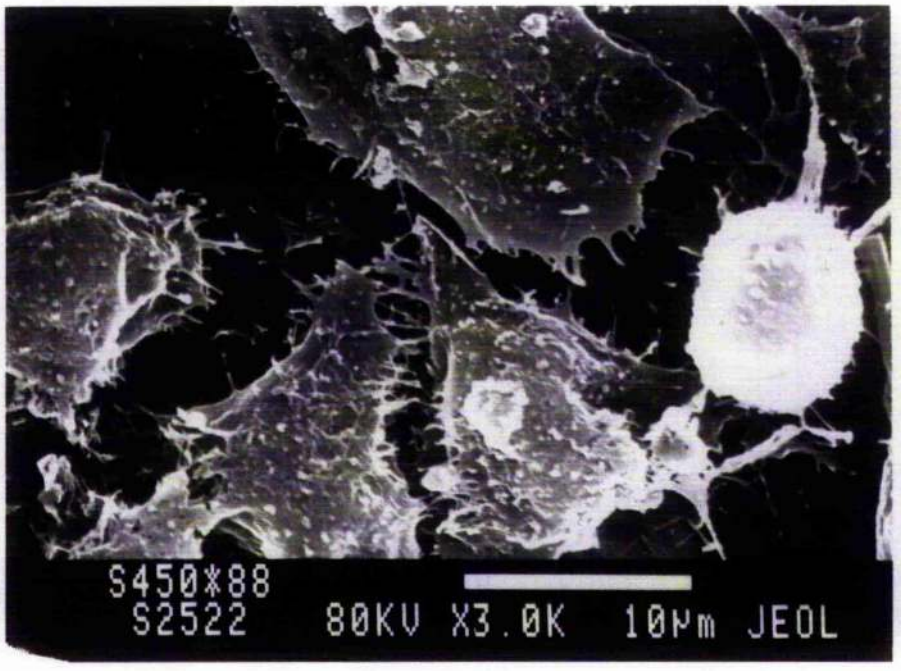
Plate 9

Scanning electron microscopy of REL-C7 cells.

A. Suspension cells cultured in MEM-O++.



B. Adherent cells cultured in MEM-O±/FBS 0.5%.



In contrast, adherent REL-C7 cells had a flat, angular appearance, often measuring greater than 20μm across. Numerous cytoplasmic extensions / pseudopodia were evident, stretching out from the cell to a distance of 3-4μm. The cell surface seemed to retain many of the small blebs evident on the suspension cells. In Plate 9B, as well as four flat angular cells, there is also one cell which has retained a spheroid appearance. A direct comparison demonstrates the profound alteration in morphology between suspension culture cells and adherent cells.

Interestingly, in Plate 9B, the two cells in the centre foreground appear to be closely associated with each other via their cytoplasmic extrusions. One can only speculate what information (if any) they are communicating.

In summary, a chance observation was made when experiments were set up to determine the lowest concentration of FBS that would support DMSO-induced erythroid differentiation. Cells maintained in medium with the FBS concentration reduced to 0.5% (rather than the standard 10%) adhered to the plastic surface of culture flasks and their gross appearance changed markedly. Several strands of evidence indicated that these changes were a result of myeloid differentiation. Both light microscopy and scanning electron microscopy suggested that the appearance of these cells was consistent with cells of the monocyte / macrophage phenotype. In addition, specific staining with α-naphthyl acetate esterase demonstrated the presence of granulation, indicative of monocytic / macrophage cells. Furthermore, when these cells were analyzed with specific monoclonal antibodies, the adherent cells showed strong expression of a macrophage marker (identified by OX-41) that was not detectable on suspension cells.

Taken together, these data provide compelling evidence that REL cells can mature along a monocyte / macrophage pathway. These cell lines were named "REL" because of their capacity to undergo erythroid differentiation on exposure to DMSO (143), REL being an acronym for Rat **Erythro**Leukaemia cells. It would appear that these cells are not restricted to the erythroid lineage and, in fact, are far more versatile.

The original leukaemia was elicited in Long Evans rats by the serial administration of the polycyclic aromatic hydrocarbon, 7,12-dimethylbenz(a)-anthracene (DMBA). The majority of the leukaemias obtained (ca. 80%) were classified as erythroleukaemia (141). The erythroid nature of this leukaemia was re-inforced by the observation that leukaemic cell lines could be established and these cell lines could differentiate along an erythroid pathway. Data presented here, however, contradict the assumption that these leukaemic cells are inherently erythroid. It may be more accurate to regard these cells as being derived from a "stem cell" leukaemia.

3.8 Cytogenetic analysis of REL-C7 cells

Tumour cells in general, and leukaemic cells in particular, can be characterised by cytogenetic abnormalities (175). It has been amply demonstrated that sites of consistent chromosomal rearrangements help to identify genes which may be critically involved in malignant transformation, and that these rearrangements themselves can subvert the normal functioning of these genes. The classic example of this is the so-called Philadelphia chromosome found in patients with chronic myeloid leukaemia (CML) (176). Haematopoietic cells from the majority of patients with CML contain a reciprocal translocation of genetic material between chromosomes 9 and 22, t (9,22). This results in a chromosome 22 that is smaller than normal, called the Philadelphia chromosome (Ph') (named after the city in which this discovery was made).

Cytogenetic analyses of REL-C7 cells were set up to determine if any consistent chromosomal rearrangement could be detected (Section 2.21). The major chromosomal rearrangements detected were:-

(a) deletion, and presumptive loss, of a segment from the long arm of chromosome 1, namely (q51-55), which has no known mouse or human homology (177).

(b) a major portion of chromosome 3 (q12-43) had translocated to chromosome 1 which had lost its terminal (q51-55) region (see(a)).

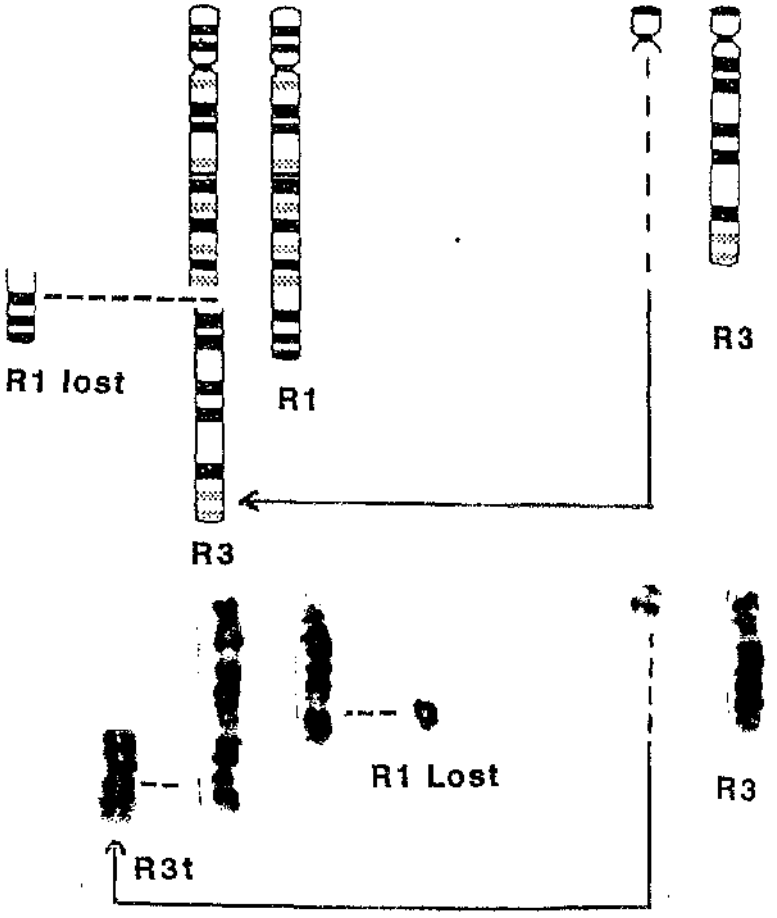
See Figure 25.

Comparative mapping of genes between rat, mouse, and humans indicates some areas of homology. Although rat chromosome 1 (q51-55) has no known homology with any mouse or human chromosomes, rat chromosome 3 has a high degree of homology with mouse chromosome 2 and a considerable length of human chromosome 9 (in fact, 9q 13-34). The breakpoint on rat chromosome 3 has been mapped to position q12 (177). Interestingly, this area also contains the proto-oncogene, c-abl.

Earlier, by way of introduction, brief reference was made to the reciprocal translocation t (9,22) often detected in CML. More precisely, this translocation occurs at t (9,22)(q34, q11), i.e. the breakpoint on chromosome 9 is at band q34, and the breakpoint on chromosome 22 is at band q11.In humans, the c-abl proto-oncogene has been mapped to chromosome 9 (q34). Thus, as a result of this translocation, c-abl is transferred from chromosome 9 at band q34 to the bcr gene (breakpoint cluster region) at band 22q11. The formation of this hybrid gene is believed to be the crucial event in the malignant transformation of the haematopoietic (stem?) cell that subsequently manifests itself as CML.

It is tempting to speculate that a similar mechanism is in operation with REL-C7 cells. It may be that the translocation of a segment of chromosome 3 (comprising the rat equivalent of c-abl) to chromosome 1, juxtaposes c-abl next to another gene or regulatory domain that can subvert the normal expression or activity of c-abl, conferring a proliferative advantage on these cells.

Figure 25 **The major chromosomal rearrangements detected in REL-C7 cells.**



SECTION 4

DISCUSSION

Almost thirty years ago, Huggins and Sugiyama (141) reported the induction of leukaemia in rats following repeated doses of a potent carcinogen, DMBA. The most common type of leukaemia that was produced appeared to be erythroleukaemia. Subsequently, Kluge et al demonstrated that permanent cell lines (i.e. REL cells) could be established from leukaemic rat tissues and that these cells could be induced to differentiate along an erythroid lineage following exposure to DMSO (143).

In the intervening years, these REL cells have been largely neglected as tools to investigate the phenomenon of differentiation. To the best of the author's knowledge, the data submitted in this thesis represent the fullest characterisation to date of these erythroleukaemic cells.

The cell lines described were established by removing the spleen from a leukaemic rat and perfusing it with culture medium to release cells into free suspension. These suspensions gave rise to permanent cell lines. This is in contrast to an earlier report in which cell lines were established by injecting newborn rats with blood from leukaemic rats (143). Tumours developed in the newborn rats, and cells from these tumours were set up in culture medium and gave rise to permanent cell lines.

Suspension cultures were optimally maintained in culture medium supplemented with foetal bovine serum (FBS) at a final concentration of 10% (v/v). Although REL cells did not appear to be particularly fastidious in their FBS requirement, batches of FBS were pre-screened to identify the most suitable ones. In addition, the concentration of FBS could be varied between 5 and 20% without any deleterious effect on cell growth.

REL cells were readily grown in suspension culture and the time taken for the number of cells to increase by a factor of 2 (doubling time) was between 12 and 14 hours. This short doubling time is indicative of the high level of proliferation of these

cells. For eukaryotic cell lines, the doubling time is unlikely to be shorter. For example, murine erythroleukaemic cells (MELC / Friend cells) have been widely studied and their doubling time is of the order of 14 hours (178). However, other established cell lines have a much slower rate of cell division. The HL-60 promyelocytic cell line grow in suspension culture with a doubling time anywhere between 20 and 48 hours depending on the subline (179, 180). Similarly, K562 cells can have a doubling time between 24 and 32 hours (181, 182). Perhaps a rather extreme example is the HL-92 cell line (immature myeloid / monocyte) which has a doubling time of 90 - 100 hours (183).

Like other cell lines, it is possible to propagate and study REL cells in systems other than suspension culture. A particularly useful technique is the so-called clonogenic assay which exploits semi-solid culture media to immobilise cells to a discrete position in the culture system. Thus, the behaviour of individual cells and their progeny (i.e. clones) can be investigated. Agar gel has been used to isolate clones of HL-60 cells (184), or, alternatively, methylcellulose has been used to clone K562 cells (185).

REL cells could be readily cloned in methylcellulose-containing semi-solid culture medium. By this technique, the cloning efficiency of REL cultures was of the order of 60%. That is, in any population of REL cells, some 60% were proliferating and undergoing cell division to give rise to progeny. The remaining 40% of cells, presumably, failed to grow. This is an interesting phenomenon. The cells in this non-proliferating subpopulation were not obviously dead. For example, by Trypan Blue exclusion, cells maintained in MEM-O++ under standard conditions were consistently >99% viable. Despite this high viability, a significant proportion of the cells seemed unable to divide. The one caveat to this, is the reliability of the Trypan Blue exclusion test. The basis of the test is that live, viable cells will have an intact, functional outer membrane which prevents the penetration of the Trypan Blue dye into the interior of the cell. Conversely, dead or dying cells are likely to have an outer membrane whose integrity has been compromised in some way by whatever cellular insult caused the

fatal damage in the first place. In this case, Trypan Blue would be able to penetrate these cells. Thus, a cell which stains blue could be predicted, with some confidence, to be dead. On the other hand, the exclusion of the dye does not necessarily indicate that the cell is fully functional. It only indicates that the outer membrane is sufficiently intact to prevent penetration of the dye. In other words, it may indicate that a particular cell is viable, but it does not tell us "how viable" that cell is.

The cloning efficiency of REL cells was calculated from cultures set up in semi-solid medium rather than the standard liquid suspension culture. Could it be that this semi-solid culture system is suboptimal and that this may account for the inability of some of these cells to divide? From the single cell analyses (Section 3.5.1), this is unlikely. When REL cells were plated out at limiting dilution in liquid culture into the wells of a tissue culture plate, the resulting cloning efficiency (53-62%) was very close to the cloning efficiency obtained with the methylcellulose assay (55-59%) (Section 3.2). It would appear, therefore, that with the in vitro culture techniques available, a significant proportion of the cells lose the ability to divide. Nevertheless, this clonogenic assay is highly sensitive as demonstrated by the limiting dilution experiments. Even when cells were plated out at a dilution of 0.5 cell per well, the resulting cloning efficiency was similar to experiments when $1x10^3$cells were plated out in methylcellulose. Thus, these cloning techniques can be used to observe cell behaviour at the level of single cells.

Obviously, all these cell lines were derived from aberrant tissue from multicellular living organisms (i.e. rodents, humans). Although these cell lines are normally studied *in vitro* in tissue culture systems, it is nevertheless interesting to see if they have retained the crucial ability that allowed their isolation and propagation in the first place, namely their ability to grow in an dysregulated fashion *in vivo*. Clearly, with human cell lines, this option is not available. However, the malignant nature of cell lines can be retained. MEL cells can produce tumours when implanted into DBA/2 mice, the strain in which the tumours originated. Titration studies indicated that an inoculum of 500 cells would produce large tumours within 3 weeks (178). In a

similar fashion, REL cells also retain their tumourogenic potential. The potency of the cells is demonstrated by the TD_{50} (i.e. the number of cells which will produce tumour in 50% of the recipient animals) which is remarkably low at a dose of 12 cells per recipient. Again, this indicates that the *in vivo* tumourogenic assay is a sensitive technique for measuring the malignant nature of REL cells.

Normal haematopiesis is a heirarchy whereby primitive stem cells can give rise to mature blood cells (186). These primitive cells consist of multipotential haematopoietic stem cells which possess extensive self-renewal capabilities (i.e. can give rise to new stem cells) and which can also generate progenitors that are programmed to differentiate. In turn, these progenitors become committed to a single lineage while retaining, at least some, proliferative ability. The progeny of these committed cells are mature blood cells which have specialised functions but have lost the ability to proliferate and have a limited lifespan. There is, therefore, a reciprocal relationship between maturation and proliferation in that the most primitive cells have extensive proliferative potential, while the most mature cells are incapable of cell division. It is the perturbation of this balance between maturation and proliferation that is the crucial event in leukaemogenesis. Thus, in normal haematopoiesis, as a particular cell differentiates towards a more mature phenotype, it will concommitantly lose its self-renewal potential. In leukaemia, however, there appears to be an uncoupling of maturation and proliferation so that immature cells, which are normally transitory, retain their self-renewal ability and accumulate in apparent maturation arrest (187). Can this maturation arrest be reversed? If the central defects in leukaemia are basically regulatory in nature, then it might be possible to induce terminal maturation. Clearly, then, it is necessary to understand how differentiation is regulated and how these aberrant differentiation programmes might be manipulated to circumvent the maturational impasse. It has been shown that established leukaemic cell lines can be induced to differentiate and express characteristics of a more mature phenotype. These model systems, therefore, present an opportunity to devise strategies that can induce terminal maturation and thus exhaust the leukaemic clone.

In the differentiation studies with REL cells, the majority of the data reported used DMSO as the chemical inducer. In a limited investigation, several other inducers were also identified (e.g. HMBA, ATRA), but DMSO was the most effective in this system. Studies with MELC cultures allowed the identification of a broad spectrum of unrelated agents (188) some of which were strong inducers of differentiation, some of which were weak. The agents were diverse in their molecular structure, ranging from polar-planar compounds, to antibiotics, to fatty acids. It is worth noting that an inducer for one cell line is not necessarily an inducer for another cell line. For example, HMBA and DMSO are potent inducers of erythroid differentiation in MEL cells. However, both these compounds are ineffective in inducing erythroid differentiation in K562 cells (189). Conversely, hemin and butyric acid are potent inducers of K562 cells, but are, at best, weak inducers of MEL cells (188, 189). In addition, there are agents, such as bleomycin or 6-thioguanine, that are potent inducers for both K562 cells and MEL cells. These cell lines are all regarded as erythroleukaemic. The HL-60 is a promyelocytic leukaemia cell line. Some agents that are capable of inducing erythroid differentiation in K562 and/or MEL cells are also capable of inducing granulocytic differentiation in HL-60 cells, e.g. DMSO, HMBA, 6-thioguanine (190).

In Section 3.4 of this work experiments were set up to investigate chemically-induced erythroid differentiation of REL-C7 cells. In the majority of the studies, DMSO was used as the inducer. When REL-C7 cells were incubated with DMSO, many of the cells differentiated to a more mature, erthroid morphology. This was accompanied by a decline in the proliferative ability of these cells as demonstrated by monitoring the changes in cell numbers over a period of 5 days. Whereas control REL cells (i.e. no DMSO) had an average doubling time of 12 hours, cells cultured in the presence of DMSO had an extended doubling time. Over the first 48 hours of DMSO exposure the doubling time was 16 hours and this was further extended so that over a later 36 hour period (Time 60hr → Time 96hr) the cell numbers increased only

modestly from 0.52 x 10^6/ml to 0.70 x 10^6/ml. Clearly, as these cells are induced to differentiate, their proliferative ability declines.

Alternative means for studying the proliferation of REL-C7 cells were the clonogenic assay and the tumourogenic assay. The clonogenic assay allows the observation of individual cells. Exposure of cells to DMSO had a profound effect on the ability of cells to form clones. After as little as 6 hours exposure to DMSO, a significant reduction in cloning efficiency was detected. This effect continued so that by 48 hours of exposure to DMSO, the cloning efficiency had declined to 0.325%. Along with the data from the growth curves, these results indicate that DMSO exerts its influence on REL cells immediately (i.e. no lag time). This proliferative down-regulation would appear to be an early event in DMSO-induced erythroid differentiation.

From the tumourogenic assay, it was shown that REL-C7 can retain their malignant potential. Indeed, it was calculated that an inoculum of as little as 12 cells would elicit tumour in 50% of the recipients. Groups of Long Evans babes received either control cells (no DMSO) or differentiated cells. Different groups received different doses, ranging from 1 x 10^4 per recipient to 1 x 10^1 per recipient (Table 14). Although some 12 control cells seemed able to produce tumour in 50% of recipients, a dose of 1 x 10^4 DMSO-treated cells elicited tumour in only 43% of recipients. This represents a reduction in tumourogenic potential of approximately 3 orders of magnitude.

If all the tumourogenic data from the controls are combined, 10 undifferentiated cells would be expected to produce tumour in 50% of recipients. In those babes that received 1 x 10^4 differentiated cells, a similar rate of tumour incidence was obtained, namely 43%. These data indicate that only a small proportion of DMSO-treated cells retained their tumourogenic potential. It may be that an inoculum of 1 x 10^4 differentiated cells contains of the order of 10 cells which still retain their ability to produce tumour, i.e. 1 cell in 1,000. This tends to corroborate the

data from the clonogenic studies. Following exposure to DMSO for 48 hours, the cloning efficiency dropped to 0.325%, i.e. 3 cells in 1,000.

Thus, these data indicate that as DMSO induces REL-C7 cells to differentiate to phenotypically mature erythroid cells, there is a reciprocal loss of proliferative potential.

In these studies of DMSO-induced differentiation, down-regulation of proliferation seemed to be an early event . Some of the effects of DMSO could be detected after 6 hours exposure. This was in contrast to the length of time taken to obtain significant levels of benzidine positive (B+ve) cells. Generally, B+ve cells were first detected after some 72 hours of incubation in the presence of DMSO, rising to a maximum at about 96 hours. These early events were analyzed by investigating the process of **commitment** to differentiation (commitment is defined as the ability to continue differentiation in the absence of inducing agent).

Obviously, the first observation was that REL cells could indeed become irreversibly committed to erythroid differentiation following exposure to DMSO for a brief period of time (Tables 17A-G and Summary). Cells were incubated with DMSO for various periods of time. They were then washed to remove any residual DMSO and set up in inducer-free medium. Two trends were observed. First of all, as the length of exposure to DMSO increased, then so too did the proportion of B+ ve cells. As little as 8 hours exposure to DMSO was sufficient to induce a small, but significant, proportion (2.1% B+ve) of the cells to differentiate. This increased progressively until, after 48 hours of exposure to DMSO, all the cells analyzed were B+ve. Similar results have been obtained with MEL cells and HL-60. With MEL cells, a period of about 12-18 hours exposure to inducer (in this case, HMBA) was required before committed cells begin to appear (191), while commitment of HL-60 cells to terminal differentiation was initiated within 8-18 hours after exposure of the cells to either DMSO or retinoic acid (192).

Secondly, with increasing exposure to DMSO, there was an increase in the incidence of colonies that contained B+ve cells. In control cells that had not been

exposed to DMSO, 14 colonies were produced, 3 of which contained some B+ve cells (3/14 = 21%). This is in good agreement with the results from Section 3.5.2 (Spontaneous erythroid differentiation) where, in the comparable experiment, 23% (5/22) of the colonies contained some B+ve cells (Table 15 Summary). After 4 hours exposure to DMSO, there was no significant change in the proportion of colonies containing B+ve cells (22%) (4/18). However, after 8 hours exposure to DMSO, the proportion of colonies containing B+ve cells increased dramatically to 56% (10/18). This trend continued. After 16 hours of DMSO, 68% (13/19) of colonies contained B+ve cells; after 24 hours of DMSO, 78% (14/18) of colonies contained B+ve cells; after 32 hours of DMSO, 96% (22/23) of colonies contained B+ve cells; and after 48 hours of DMSO, every colony contained B+ve cells. These data demonstrated the continual recruitment of REL cells to a differentiative programme with increasing exposure to DMSO.

These commitment studies with REL cells were done in limiting dilution assays. The advantage of this was that it allowed the behaviour of single cells to be observed, i.e. it was possible to study all the progeny from a single cell following exposure to DMSO. Considerable heterogeneity was observed in these colonies (Tables17A-G, Table18). Some colonies contained no B+ve cells; others contained a mixture of B+ve and B-ve cells; others were exclusively B+ve. From the cells that received the shorter DMSO exposures (0 hr, 4 hr, 8 hr) a number of colonies were obtained which contained B+ve cells. However, none of these colonies were exclusively B+ve. All of these colonies were of the mixed type. It was only with increasing exposure to DMSO that colonies that were uniformily B+ve started to appear. For example, after 16 hours exposure to DMSO, of the 13 colonies that contained B+ve cells, only 2 were exclusively B+ve (15%). After 24 hours exposure to DMSO, of the 14 colonies that contained B+ve cells, 6 were exclusively B+ve (43%). This trend continued. After 32 hours exposure, 22 colonies were obtained, of which 17 (77%) were exclusively B+ve. After 48 hours exposure, all colonies were exclusively B+ve. These data demonstrated the continuing commitment of REL cells.

Thus, increasing exposure to DMSO not only induces more cells to produce differentiated progeny (increase in the number of colonies containing B+ve cells), but also induces cells to produce progeny that are increasingly B+ve (increase in the proportion of B+ve cells within any one colony).

A further demonstration of the down-regulatory effect that DMSO has on proliferation can be seen from an analysis of the cell numbers in individual colonies (Table 17 Summary). Cells that were not exposed to DMSO and cells that were exposed to DMSO for 4 hours both tended to produce large colonies with a median number of cells per colony of ~1,000. As the DMSO exposure time increased, then the median number of cells per colony decreased. Thus, cells exposed to DMSO for 8 hours produced colonies which had a median number of cells per colony of ~625. Cells exposed to DMSO for 16 hours produced colonies with a median number of cells per colony of 156. This inhibition of proliferation continued with the median number of cells per colony declining to 50 cells (24 hour exposure), 4 cells (32 hour exposure), and 2 cells (48 hour exposure).

Another interesting aspect of these studies is that, under these culture conditions, committed cells can give rise to both differentiated and undifferentiated progeny. From control cells that were not incubated with DMSO, 3 colonies (out of a total of 14) were obtained that were of the mixed phenotype. Since there was no DMSO exposure, one can safely assume that these cells differentiated spontaneously. From cells that were exposed to DMSO for 4 hours, 4 mixed colonies (out of a total of 18) were obtained. The differentiated cells in these colonies may have been committed by the presence of DMSO, or alternatively, they may have differentiated spontaneously. Cells exposed for 8 hours produced 10 mixed colonies (out of a total of 18). It is possible that some of the differentiated cells in these mixed colonies may have arisen spontaneously, but it is unlikely that spontaneous differentiation occurred in all 10 mixed colonies. At least some of these differentiated cells must have been induced by exposure to DMSO. So, individual cells that have been exposed to DMSO can produce progeny that are differentiated as well as progeny that have failed to

differentiate. Similar heterogeneity of colony morphology has been reported with MEL cells (191).

The down-regulatory effect that DMSO had on REL proliferation was analyzed further by assessing the rate of DNA synthesis. This was done by incubating REL cells with DMSO for various periods of time and then measuring the incorporation of ^{3}H-thymidine into cellular DNA. Thus, those cells that are proliferating rapidly will have a high rate of DNA synthesis, and will therefore incorporate relatively large amounts of ^{3}H-thymidine over a defined period of time. On the other hand, those cells in which proliferation has been down-regulated will have a slower rate of DNA synthesis, and will, therefore, incorporate a correspondingly lower amount of ^{3}H-thymidine over the same time interval.

Further corroboration was obtained that even brief exposure to DMSO can inhibit proliferation as measured by the amount of DNA synthesis. In two of the three experiments set up, a significant reduction in proliferation was detected after as little as 2 hours exposure to DMSO. In the other experiment, there was a reduction in DNA synthesis, but not significantly so (Table 22). After 4 hours exposure to DMSO, all three experiments showed at least a significant reduction in DNA synthesis, and in two out of three experiments the difference was highly significant. After 6 hours exposure to DMSO, all three experiments showed a highly significant difference from the control cells. Thus, these data indicate that DNA synthesis can be down-regulated after 2 hours exposure to DMSO. Previously (Section 3.5.3), it was shown that REL cells started to become committed to erythroid differentiation after a minimum of 8 hours exposure to DMSO. It would appear, therefore, that this reduction in proliferation is an early event in the process of commitment to erythroid differentiation.

When a cell divides, the two resulting daughter cells each receive the full complement of genetic material. Clearly, this requires the parental cell to duplicate the genetic material prior to cell division (mitosis). It has been known for many years that the process of eukaryotic cell proliferation can be divided up into discrete periods of

activity (193). Thus, DNA replication does not occur throughout the cell cycle, but rather it is restricted to a particular phase of it, called S-phase (S for synthetic). There is a post-synthetic, pre-mitotic period designated G_2 (G for gap). This precedes mitosis. There then follows a post-mitotic, pre-synthetic period designated G_1. Proliferating cells would proceed through G_1 into S-phase, then into G_2, and then divide, thus completing the cell cycle:- $G_1 \rightarrow S \rightarrow G_2 \rightarrow M \rightarrow G_1 \rightarrow S$, etc. In addition, under certain conditions, cells may opt out of this proliferative cycle and assume a stable, resting state in which cell division has ceased. Such cells are said to have entered a "G_0 phase" of the cell cycle.

REL cells were exposed to DMSO for various periods of time and then subjected to cell cycle analyses to try to elucidate the manner in which their proliferative status was down-regulated. Both experiments (Table 23) were in broad agreement. In normally proliferating cells (i.e.MEM-O++ without DMSO), at any one time, the majority of cells were in S-phase (69% and 83% respectively) and relatively few cells were in G_0/G_1 or G_2+M. As cells were exposed to DMSO, a clear pattern developed. Generally speaking, with increasing exposure time to DMSO, there was a corresponding decrease in the proportion of cells in S-phase, i.e. the number of cells synthesizing DNA declined. In the first experiment, a reduction in the proportion of cells in S-phase could be detected after only 1 hour of DMSO exposure. However, the major decrease occurred between 4 and 6 hours exposure when the proportion of cells in S-phase dropped from 58% to 41%, with the trend continuing down to 35% (8 hours exposure) and 29% (24 hours exposure). A similar pattern was seen in the second experiment. Again, a reduction in the proportion of cells in S-phase could be detected after as little as 1 hour exposure to DMSO. However, in this experiment the major reduction was seen between 1 and 2 hours of DMSO exposure with the proportion of S-phase cells declining from 76% to 46%. This general trend continued so that after 24 hours exposure to DMSO, only 27% of cells were in S-phase.

As the proportion of S-phase cells declined, a corresponding increase in the proportion of cells in G_0/G_1 could be detected. In the first experiment, the proportion

of cells in G_2+M remained fairly constant. Thus, any decline in S-phase cells could largely be accounted for by the corresponding increase in G_0/G_1 cells. For example, between 4 and 6 hours DMSO exposure, there was the greatest decrease in S-phase cells (58% to 41%) which directly coincided with the greatest increase in G_0/G_1 cells (30% to 47%). With increasing exposure times to DMSO, the proportion of cells in G_0/G_1 continued to rise to 60% (24 hours). In the second experiment, although this reciprocal relationship between S-phase cells and G_0/G_1 cells was still evident, the data were not quite so clear-cut. Although the major reduction in S-phase cells occurred between 1 and 2 hours of DMSO exposure (76% to 46%), the corresponding major increase in G_0/G_1 cells did not occur until between 3 and 4 hours of DMSO exposure (26% to 50%). This was largely due to an apparent increase in the proportion of cells in G_2+M, unlike experiment 1 in which the level of G_2+M cells remained relatively constant. Nevertheless, the same general trend was observed in that, with increasing exposure to DMSO, the proportion of cells in G_0/G_1 increased from 11% (no DMSO) to 69% (24 hours exposure).

These data corroborate the earlier findings that even brief exposure to DMSO can effect profound alterations in the proliferative status of REL cells. At a relatively gross level, it was shown by growth curves and the clonogenic assay, that cell division was down-regulated in the presence of DMSO. More precisely, DMSO seemed to inhibit cell division by inducing a reduction in DNA synthesis. More precisely still, DMSO appeared to cause this switch-off of DNA synthesis by preventing the cells from entering S-phase of the cell cycle. Instead, REL cells tended to accumulate in G_1 phase, unable to make the transition to S-phase. Clearly, if cells cannot pass through S-phase, there can be no DNA synthesis and consequently no cell division.

Understanding the regulation of the cell cycle holds the key to understanding whether a cell will continue to proliferate, or alternatively, whether it will cease dividing and become committed to differentiate along a particular lineage to produce functional end-cells. Not surprisingly, a great deal of attention has focused on events in the cell cycle to understand how inducers of differentiation exert their effects.

Probably the most widely studied system is the MEL cell (Friend cell). In the earliest reports, several groups provided evidence that the inducer (DMSO) was required to be present during at least one, and possibly more, rounds of DNA synthesis to allow differentiation to proceed (194, 195). Further investigations indicated that DMSO (and some other inducers) delayed entry of cells into S-phase of the cell cycle, thus prolonging the length of G_1 phase (196). Subsequently, Pragnell et al reported that after 12 hours incubation with inducer (butyric acid), the numbers of cells entering S- and G_2+M phases started to decline rapidly, and increasing numbers of cells accumulated in "G_1 arrest" (197). When cells were sorted on the basis of their cell cycle status it was shown that, following induction with butyrate (197) or HMBA (198), only G_1 arrested cells were committed to differentiate. Furthermore, only G_1 cells synthesized globin mRNA, suggesting that commitment to differentiation and globin mRNA accumulation are linked events. Thus, the trigger to commitment and terminal differentiation only occurs during passage of cells through G_1. A further subtlety was reported by the Glasgow group exploiting MEL cells which were temperature-sensitive (ts) for growth (199). At permissive temperatures, ts cells replicated optimally and accumulated haemoglobin in response to inducer (HMBA). However, at a non-permissive temperature the cells were arrested in G_1 and, in the presence of inducer, did not accumulate haemoglobin or globin mRNA, nor did they undergo terminal differentiation. G_1 ts cells, treated with inducer, accumulated haemoglobin when released from growth arrest at the permissive temperature in the presence, but not in the absence, of inducer. These data indicated that there was a requirement for a cell cycle-dependent event prior to commitment to differentiation. The data obtained with REL cells are in broad agreement with the MEL story, but the mechanism outlined above is not necessarily the only one.

Although some reports have indicated that commitment to differentiation occurs in G_1 phase and possibly early S-phase (194, 197), studies with HL-60 cells suggested that retinoic acid induces commitment preferentially during the S- or the S/G_2 interphase (200). Furthermore, using DMSO as the inducer, the cell cycle

pattern had no bearing on differentiation of HL-60 cells (201, 202). Thus it would appear that DMSO and retinoic acid initiate differentiation in HL-60 cells by different mechanisms (203, 204). Similar findings have been reported with K562 cells (205). Whereas cells in late G_1 and early S phases were most sensitive to ara-C, hemin caused induction regardless of the cell cycle pattern. Indeed there was evidence that these agents could act synergistically to produce an earlier, more rapid increase in the number of induced cells, again reflecting the diverse mechanisms of action of these agents.

However, in REL cells, and other similar systems, the cell cycle status of cells appears to be the determining factor in commitment to differentiation. How then does the cell cycle influence the decision to either continue proliferation or, alternatively, to differentiate to a more mature phenotype with subsequent loss of proliferative potential?

Over the last few years, great progress has been made in our understanding of the regulation of the events in the cell cycle. Due to the delay in the submission of this thesis, some of the aspects of the REL studies presented have been overtaken somewhat by these developments. Nevertheless, it is important to put the REL data in perspective with current thinking.

Control of the cell cycle involves an integrated cascade of regulatory proteins which includes cyclins and cyclin dependent kinases (CDK). Cyclins are synthesized and activated in a cell cycle-dependent manner and function as regulatory subunits of CDK's. CDK's phosphorylate transcription factors to control cell cycle-regulated genes and proteins that support proliferation. For example, growth factor-induced cell proliferation is mediated by D-type cyclins (D1, D2, D3) which are synthesized in G_1 in response to growth factor stimulation (206). While the levels of cyclins tend to oscillate with the phases of the cell cycle, CDK's are present at similar levels throughout. The activities of these CDK's are controlled by these specific cyclins and by a series of recently described cdk inhibitors (CDKI) (207, 208). In particular, in cellular proliferation, the initiation of the cell cycle is mediated largely by complexes

of the D-type cyclins with CDK4 and CDK6 (209). Thus, interference with cyclin D1 function by either antibodies or antisense oligonucleotides can prevent cells from progressing into S-phase (210).

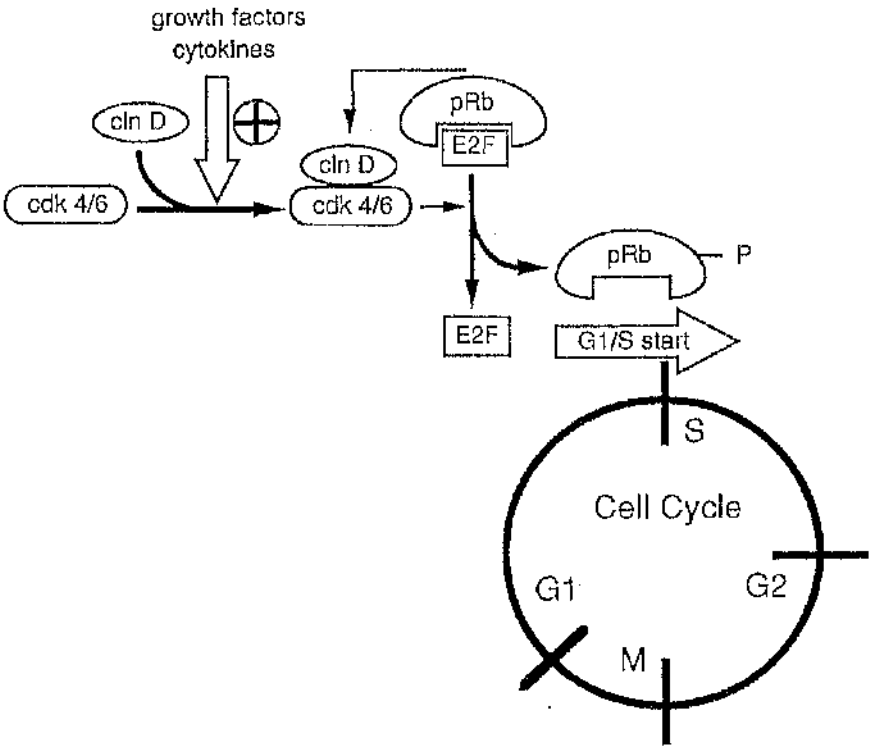
It has recently been shown that D-type cyclins can complex with CDK4 and CDK6 and bind to the retinoblastoma gene product pRb (211). The retinoblastoma gene is regarded as a tumour suppressor gene (or anti-oncogene) in that mutation or loss of both copies of the gene is associated with a number of human cancers (212). pRb is thought to have a pivotal role in cell cycle progression and cell division. During early G_1, the hypophosphorylated form of pRb binds and inactivates the E2F transcription factor, resulting in suppressed transcription of E2F-regulated genes (213, 214). Among these are the genes for DNA polymerase α, dihydrofolate reductase, and thymidylate synthetase which are required for DNA replication (215). D-type cyclins can form complexes with CDK4 or CDK6 to phosphorylate pRb. When pRb is in the hyperphosphorylated form, it dissociates from E2F, allowing the released E2F to promote the transcription of genes associated with DNA replication (Figure 26). Subsequent progression through G_1 into S-phase is controlled by another complex, Cyclin E / CDK2 (216).

In MEL cells undergoing HMBA-mediated commitment to differentiation, hypophosphorylated forms of pRb accumulate, accompanied by an increasing amount of total pRb (217). These hypophosphorylated forms of pRb are likely to bind E2F, thus preventing the transcription of a range of genes associated with DNA replication, i.e. proliferation would be down-regulated. These data suggested that pRb may play a role in the recruitment of cells to terminal differentiation. Subsequently, it was shown that HMBA-induced differentiation of MEL cells involved a rapid decrease in the level of CDK4 protein, thus suppressing the kinase activity of CDK4 (218). In addition, the level of cyclin D3 was increased by HMBA, and this protein formed complexes with pRb and E2F as the cells were progressively committed to differentiation. Further evidence for the critical role of CDK4 was supplied by DNA transfection studies which showed that forced overexpression of CDK4 protein

Figure 26

Schematic representation of the interrelationships between cell cycle regulatory proteins (Abridged from Ref. 209) .

Abbreviations : cyclin D (cln D); cyclin dependent kinase (cdk); retinoblastoma protein (pRb); transcription factor E2F (E2F).



suppressed the recruitment of MEL cells to terminal differentiation. These observations suggest that chemically-induced differentiation of MEL cells involves the modulation of cyclins, CDK's, transcription factors (e.g. E2F), and molecular intermediates (e.g. pRb) which together can down-regulate proliferation and direct the expression of differentiation-specific genes.

In addition to these proliferative alterations, commitment and differentiation elicited some gross changes to REL cells. For example, REL cells undergo a progressive reduction in cell size with increasing exposure to DMSO, as measured by flow cytometry. Although there appeared to be a decrease in cell volume after as little as 8 hours exposure to DMSO, it was only after 24 hours exposure that a significant reduction was observed (Figure 15). A linear relationship was obtained between the duration of exposure to DMSO and the mean cell size. A similar decrease in cell size during differentiation has been reported with HL-60 cells (219) and with MEL cells (220). In particular, in MEL cells the changes in cell size appeared to follow a bi-phasic pattern, with the first volume reduction occurring during the initial 10 hours of DMSO exposure (i.e. early), and the second volume reduction occurring after approximately 60 hours of DMSO (i.e. late). This was corroborated and extended by Zucker et al who also reported a correlation between the decrease in mean cell volume and the increase in the proportion of G_1 cells in the early time period (221). In their hands, these changes in cell volume were a rapid monitor to determine the early events of the differentiation process. Unlike this MEL differentiation, REL differentiation did not produce any evidence for a bi-phasic pattern of cell volume reduction, although, like the MEL system, a reduction in cell volume could be observed after a brief exposure to DMSO.

Scanning electron microscopy (SEM) was performed on induced REL cells to see if any further gross morphological changes could be detected. REL cells, proliferating under standard conditions, revealed a cell surface that had many well-defined microvilli. Some short ridges and some ruffles seemed to run across the cell surface. After 24 hours of exposure to DMSO, although some cells were identical to

the control cells, other cells presented a surface that was markedly altered. These altered cells did not have the microvilli and ridges of control cells, but rather they had a generally smooth surface appearance with pores and channels discernible. This trend continued with increasing exposure time to DMSO. After 48 hours of DMSO exposure, virtually all the cells had acquired this smooth surface appearance. It has previously been demonstrated in this work that, after 48 hours of exposure to DMSO, REL cells are largely non-proliferative. Uninduced, non-proliferating REL cells were also analyzed by SEM. They, too, had a generally smooth appearance with pores and channels on the surface. There was a remarkable resemblence between these uninduced, non-proliferating cells and the DMSO-induced, non-proliferating cells. These surface alterations occur over the same period of time as do the proliferative changes. The appearance of the more mature phenotype (e.g. accumulation of haemoglobin) is a much later event. Thus, these profound alterations to the cell surface may reflect the down-regulation of proliferation and the cells entering a proliferative stationary phase, rather than any maturational progression.

Similar SEM studies have been reported for MEL cells (222). Cells taken from the logarithmic phase of growth had short microvilli scattered uniformly around the cell with some ruffles evident. After 48 hours of DMSO exposure, the cells had a blebby appearance. With cells that had differentiated further (e.g. 5 days of incubation with DMSO), a polarization phenomenon was seen whereby one half of the cell was blebby while the other half, which was separated by an equatorial cord, had a smooth membrane. This process resembled the enucleation process of untransformed erythroid cells.

This dissection of the process of differentiation of REL cells allowed a sequence of events to be proposed (Figure 16). After 4 hours exposure to DMSO, a significant reduction in DNA synthesis could be detected and cells began to accumulate in G_0/G_1 phase of the cell cycle. After 6 hours of DMSO, DNA synthesis continued to decline and cells continued to accumulate in G_0/G_1. A significant number of cells were no longer capable of cell division as demonstrated by the

reduction in cloning efficiency. After 8 hours of DMSO, some cells had now become irreversibly committed to erythroid differentiation. As the duration of DMSO exposure increased, then the switch-off of proliferation became more pronounced and the number of committed cells correspondingly increased. Gross morphological changes could now be detected. A decrease in cell volume could be measured after 24 hours of DMSO and this was accompanied by the appearance of cells with a smooth surface rather than a surface with microvilli and ridges. The cells continued to decrease in size and by 48 hours of DMSO virtually all the cells had acquired a smooth surface. By 72 hours of DMSO, the first haemoglobin-producing cells appeared and these numbers rose to a maximum after 96 hours of DMSO.

To try to identify what biological activities were involved in the process of commitment to differentiation, attempts were made to adapt REL cells to a chemically-defined medium and then induce them to differentiate by the addition of known growth factors/cytokines. In effect, this meant propagating REL cells in serum-free medium. REL cells were readily adapted to serum-free conditions and their growth kinetics were only slightly inferior compared to the usual serum-containing medium. Attempts to induce REL cells to differentiate in serum-free medium were invariably unsuccessful. It appeared that there was some activity in serum that was required for differentiation to proceed.

In fact, further investigation revealed that this serum activity was required for **commitment** to differentiation. This was shown by "transfer-out" experiments. Briefly, REL cells were incubated with DMSO for 24 hours, transferred to DMSO-free medium, incubated for a further 72 hours (i.e. 96 hours in total), and then assessed for haemoglobin production by the benzidine reaction. These transfers were carried out between different combinations of serum-containing medium and serum-free medium. For example, cells were exposed to DMSO in serum-containing medium, and then transferred to serum-containing medium that did not contain DMSO. These cells subsequently generated high numbers of B+ve cells, indicating

that extensive commitment had occurred in the initial 24 hour culture in the presence of DMSO (and serum).

A further combination was set up in which cells REL cells were again cultured with DMSO for 24 hours in serum-containing medium. On this occasion they were transferred to serum-free medium that did not contain DMSO. Although previous experiments had indicated that serum-free conditions could not support differentiation, these transferred cells did produce significant numbers of differentiated progeny (Figure 23). The proportion of B+ve cells in this second experiment (serum-containing → serum-free) was less than the proportion of B+ve cells found in the first experiment (serum-containing → serum-containing). This difference probably reflects that, after 24 hours exposure to DMSO, although a significant proportion of the cells had become committed to differentiate, commitment was still incomplete, i.e. some cells had still not been recruited to commitment. If the exposure time to DMSO had been extended to, say 48 hours, then it is likely that more cells would have become committed and, therefore, a greater proportion of the cells would have been B+ve. In any event, these data demonstrate that serum is not necessarily required for the entire process of differentiation, but rather it is required only over the crucial commitment period. At least to some extent, once cells have been committed to differentiate, then serum is longer required for the rest of the differentiation programme to be expressed.

In the final combination that was set up, cells were initially exposed to DMSO in serum-free medium and were then transferred to serum-containing medium . Only very low levels (2%) of B+ve cells were obtained. It appeared that very few cells became committed to differentiation in the initial serum-free incubation. The low level of cells that were B+ve could possibly be accounted for by some carry-over of DMSO from the initial serum-free incubation to the the subsequent serum-containing incubation, although the cells were washed thoroughly. Perhaps this combination of serum with some contaminating DMSO enabled a small number of cells to become

committed to differentiate. An additional contribution may have come from cells that had undergone spontaneous differentiation.

Although cells cultured in serum-free medium could not be induced to differentiate, they still retained the potential to do so, provided that the appropriate conditions were available. This was demonstrated by "re-adapting" cells from serum-free medium back to serum-containing medium. When these cells were incubated with DMSO, a significant proportion of the cells were B+ve. Thus, prolonged maintenance in serum-deprived conditions did not abrogate the cell's ability to express a differentiative programme.

Some preliminary experiments were set up in which serum-free medium was supplemented with a selected growth factor or combination of growth factors (IL-6, IL-11, G-CSF, M-CSF, GM-CSF, KLS, MIP-1α). At the concentrations prepared, none of the growth factors appeared to exert any appreciable effect on cell proliferation - neither stimulatory nor inhibitory (Table 27). When cells that had been adapted to these cytokine-supplemented serum-free media were incubated with DMSO, the proportion of cells that became B+ve was invariably <1% (Table 28).

These attempts to try to identify the factor(s) in serum that allowed commitment to proceed were, to say the least, speculative. It is a feature of haematopoietic regulation that cytokines tend to exert their effects in combinations, rather than in isolation. It is likely that a similar situation pertains to REL differentiation. Several, as yet unidentified, cytokines may act together in a synergistic manner to promote DMSO-induced differentiation.

Serum-free conditions have been used to study differentiation in other cell lines. MEL cells were readily adapted to serum-free medium with no impairment of cell proliferation (223). On subsequent exposure to a variety of inducers (DMSO, HMBA, hemin) a significant proportion of the cells were B+ve. In this respect, serum-free medium was inferior to serum-containing medium with all three inducers. For example, using DMSO as the inducer, serum-containing cultures were approximately 70% B+ve, while serum-free cultures were about 40% B+ve. These data suggested

that factors present in serum could play a role in modulating erythroid differentiation in MEL cells. K562 cells have also been induced to differentiate in serum-free medium (224), although some sub-lines have been selected that are unable to differentiate in serum-free conditions (225). Interestingly, in this last report, newborn bovine serum (NBS) was found to be significantly inferior to FBS in its ability to support differentiation. They concluded that K562 cells required a factor found in FBS to undergo erythroid differentiation. In contrast, REL cells were able to differentiate with a variety of sera.

The nature of the factor(s) in serum which is required to allow REL cells to differentiate remains to be resolved.

REL cells are regarded as being erythroid in nature because of their ability to differentiate along an erythroid pathway and because they possess some morphologically erythroid features. While studying the effects of reduced FBS concentrations on REL proliferation and differentiation, some REL cells underwent gross morphological changes. When cultured in medium with FBS at a concentration of 0.5% (v/v), cells became plastic adherent, rather than the usual suspension cultures observed when the medium contained FBS at 10% (v/v). These cells resembled peripheral blood, plastic-adherent, mononuclear cells; i.e.monocyte/macrophage cells. Further evidence of the monocyte/macrophage nature of these cells was provided by morphological anaysis using Wright's stain. The resulting preparations were consistent with monocyte/macrophage cells. Furthermore, cytochemical analyses with α-naphthyl acetate esterase staining gave a strong positive result, indicating the monocyte/macrophage nature of these adherent cells.

Adherent cells were also analysed with a range of monoclonal antibodies, each recognizing a specific cell surface antigen. There was little difference in the pattern of the reactions between suspension cells and adherent cells. The only difference observed was with monoclonal antibody OX-41, which recognises an antigen associated with monocyte/macrophage cells. Suspension cells were negative for this

antibody, adherent cells were strongly positive. These data provided additional evidence that these adherent cells were indeed monocyte/macrophage.

To illustrate the point further, SEM's were prepared on suspension cells and adherent cells. Striking differences were observed (Plate 9). Suspension cells were generally spheroid, about 10-12μm in diameter with surface microvilli and ridges. Adherent cells, on the other hand, were flat, angular cells measuring over 20μm in diameter with numerous cytoplasmic extensions stretching out from the body of the cell. It would seem safe to conclude that these adherent cells are indeed of the monocyte/macrophage lineage.

Similar multi-potentialities have been reported in other cell lines. As mentioned earlies, HL-60 cells can be induced to differentiate to granulocytic cells (151, 152) or macrophage-like cells (153), depending on the inducer. Furthermore, a minor population of HL-60 cells appeared to express some eosinophilic characteristics (155). This eosinophilic potential of HL-60 cells was supported by subcloning experiments which allowed the establishment of HL-60 sublines which were restricted to the eosinophilic lineage (226). Other data on the multipotential nature of cell lines is less convincing. For example, MEL cells have been reported to express both erythropoietic and granulopoietic lineage markers following induction with DMSO (227). Similarly, K562 cells have reported to express megakaryocytic antigens (228), myeloid antigens (229), and even B-lymphocytic antigens (230). This aberrant expression of surface antigens may reflect the phenomenon of lineage infidelity (93) in which individual cells co-express markers which are normally restricted to a single lineage.

The data presented here on the REL cells provides compelling evidence for their true bi-potentiality. Evidence is not restricted merely to expression of non-erythroid surface markers. Morphological and cytochemical data clearly show that these so-called erythroleukaemic cells are capable of developing along a monocyte / macrophage pathway. Perhaps this cell line should be regarded as a "stem cell" leukaemia.

The data presented in this thesis constitute the most extensive investigation to date into this rat erythroleukaemic cell line. Initial studies described the growth characteristics of the cells using a variety of culture techniques. The main thrust of this work focused on the ability of these cells to undergo erythroid differentiation following induction by a variety of chemicals. A sequence of events was described in which the proliferative potential of the cells was progressively diminished as the cells became increasingly committed to differentiation. The bi-potential nature of REL cells was also established when, under unusual culture conditions, REL cells were shown to develope into cells of the monocyte/macrophage series.

Although many characteristics of REL cells were investigated, there were some important aspects that were not addressed. Over the last decade in particular, molecular biological techniques have become increasingly powerful tools in our understanding of cellular regulation. Many genes have been implicated in the cellular dysregulation that ultimately manifests itself as neoplastic tissue. In this thesis, data were presented on cell biological aspects of commitment and differentiation. Clearly, this could have been a fertile area for molecular biological analyses of differentiation. Although REL cells have not been studied in this context, other cell lines have provided some insight into the regulation of differentiation at the molecular level.

As before, the MEL cell line is amongst the most extensively studied. Differentiation of MEL cells is associated with dramatic changes in the expression of various genes. For example, treatment with HMBA or DMSO can cause an early decrease in the expression of the proto-oncogenes c-*myc* and c-*myb*, and in the tumour suppressor gene p53, while the expression of c-*fos* increases rapidly (231, 232). The decrease in c-*myc* and c-*myb* mRNA and protein reflects decreased transcription of these genes. The down-regulation of p53 appears to be due to regulation at a post-transcriptional level. Constitutive expression of an exogenous c-*myc* cDNA inhibits inducer-mediated differentiation of MEL cells (233, 234, 235). Furthermore, constitutive expression of c-*myc* antisense RNA accelerates inducer-mediated differentiation in MEL cells (236). Cells with depressed levels of c-*myc*, due to

expression of c-*myc* antisense, arrest at an early point in G_1 phase of the cell cycle. These data imply that these changes in gene expression are among the steps necessary for inducer-mediated terminal differentiation. Likewise, constitutive expression of exogenous c-*myb* cDNA inhibits inducer-mediated MEL differentiation (237).

Similar data have come from HL-60 studies. The c-*myc* gene is markedly amplified in uninduced HL-60 cells (238), but is rapidly inactivated during granulocytic differentiation induced by retinoic acid (239).

Clearly, it would have been of interest to analyse REL commitment and differentiation at the molecular level. The patterns of expression of various proto-oncogenes could have been investigated and any subsequent information could have been integrated into the cascade of events that is initiated when REL cells are exposed to DMSO. These studies could have been extended to include REL culture in serum-free medium. Although REL cells were able to proliferate adequately in serum-free conditions, they could not be induced to differentiate. It may have been of value to investigate the pattern of proto-oncogene expression in cells cultured in serum-free medium to try to identify any significant differences from cells cultured with serum.

Preliminary cytogenetic information was provided, identifying the major chromosomal rearrangements in REL cells. Tantalisingly, the translocation between chromosome 1 and chromosome 3 involved that part of chromosome 3 that contained the c-*abl* proto-oncogene. One could only speculate that this translocation might subvert the normal function of c-*abl* and thus contribute to the proliferative dysregulation of REL cells. Molecular analyses may have been able to identify an altered form of the c-*abl* gene.

An exciting area of biological research is the regulation of cell cycle events. This, too, would merit some molecular investigation. As well as looking at the modulation of oncogene expression during commitment and differentiation, analyses of the expression of the cyclins and cyclin dependent kinases would provide a fascinating insight into mechanisms that subtly control the cell cycle.

With a greater understanding of the regulation of differentiation, it may be possible, in the fulness of time, to apply these experimental findings to devising strategies that may have some real clinical impact in the treatment of haematological malignancies.

SECTION 5

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