THE BIOLOGY OF HAIRY-CELL LEUKAEMIA, WITH PARTICULAR REFERENCE TO α-INTERFERON

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by Kathleen June Till. May 1993.

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Declaration

I declare that this thesis entitled 'The biology of hairy-cell leukaemia: with particular reference to α-interferon' is entirely my own work.

Portions of this work have formed the basis of two publications;

1. **Till, K. J. & Cawley, J. C.** Phenotypic changes on hairy cells exposed *in vitro* to interferons: a quantitative FACS study. *Br. J. Haem.* **72:**378-386.

2. **Till, K. J., Lopez, A., Slupsky, J. & Cawley, J. C.** *C-fms* protein expression by B cells, with particular reference to the hairy cells of hairy-cell leukaemia. *Br. J. Haem.* **83:** 223-231.

In addition, the work presented in Chapter 3 form the basis for another potential publication.

ABSTRACT

Since its first usage in 1983 α-interferon (IFN) has been employed in the treatment of hairy cell leukaemia (HCL) with a high degree of clinical efficacy (Quesada *et al* 1983; 1984). As this laboratory was involved in the first UK trial of αIFN (Worman *et al* 1985), our attention was directed at the mechanisms responsible for the αIFN induced remission in HCL. At the time work on this part-time thesis began (1986), investigations in this laboratory had largely shown that αIFN does not act by indirectly promoting hairy cell (HC) killing by natural killer (NK) cells (Griffiths & Cawley 1987). In addition experiments in progress in the Department, at the time, indicated that other killer cells were not involved in HC killing (Griffiths & Cawley 1988). Attention was therefore focused on the direct effects of αIFN in HCL; the initial aim of this thesis was to examine what these direct effects might be.

The effect of αIFN on a large range of HCs' surface antigens was therefore investigated. These antigens were chosen either because they are expressed by HCs, or on the activation/differentiation of normal B cells. Although αIFN caused both increased and reduced HC antigen expression, the pattern of these changes made interpretation difficult; however, the results could have been indicative of partial HC activation/differentiation. The effects of αIFN on the surface phenotype of HCs are reported in Chapter 1 of this thesis.

The proto-oncogenes *c-myc* and *c-fos* are both involved in the signalling pathways for activation (*c-myc* and *c-fos*) and differentiation (*c-fos*), and are regulated by αIFN. A study of the effects of αIFN on these two oncogenes was therefore undertaken in order to establish whether the results of Chapter 1 were indicative of HC activation or differentiation. However, αIFN treatment had no effect on the expression of the *c-myc* and *c-fos* proteins in resting HCs, although the cytokine induced both a reduction in the expression of these proteins and growth arrest in HCs that had been stimulated to proliferate. These results are reported in Chapter 2.

Although the results of Chapter 2, together with those of other workers at the time, suggested that αIFN was anti-proliferative in HCL, the nature of the *in vivo*

proliferative signal to HCs was unknown. The third chapter of this thesis is an investigation of the potential *in vivo* signals for HC growth. At the time of this study it was reported that the cytokine tumour necrosis factor (TNF) α induced HC proliferation; however it was not clear whether the cytokine was acting directly on HCs. The results presented in Chapter 3 indicate that TNFα does indeed directly stimulate HC proliferation. TNFα was also shown to be essential for the proliferation of HCs induced by accessory cells; in addition binding of HCs to these accessory cells via the leukocyte integrins was shown to be important. The anti-proliferative effects of αIFN were also seen with this physiological stimulus.

Although HCs are activated B cells they posses features found predominantly on cells of other lineages, in particular monocytes/macrophages. The maintenance of the monocytoid state has been seen to be due to the receptor ligand combination of the *c-fms* protein and the cytokine macrophage colony stimulating factor (M-CSF); this prompted an investigation of HCs to see if the monocytoid features of these cells could be attributed to the expression of this oncogene. Chapter 4 shows that HCs do indeed express the *c-fms* protein and, although the *c-fms* protein is active, binding of M-CSF to its receptor did not induce HC proliferation or differentiation, or alter HC morphology, phenotype or survival; changes that are seen on monocytes in response to M-CSF treatment.

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INTRODUCTION

The initial aim of this thesis was to investigate the mechanisms by which αIFN acts in HCL. Although αIFN treatment of HCL increases the relative number of NK cells (Foon *et al* 1986a), these cells are not involved in HC killing (Griffiths & Cawley 1987). Experiments in progress in the laboratory at the time work on this thesis started also ruled out the possibility that αIFN acted indirectly on HCs by promoting their killing by other cell types (Griffiths & Cawley 1988). Attention was therefore focused on the direct effects of αIFN on HCs.

At the time work on this thesis began I was responsible for setting up the FACS in the Department; in addition there was a degree of interest in the literature on the influences of αIFN on the surface antigen expression (Baldini *et al* 1986), and the protein synthesis of HCs (Samuels *et al* 1987a). This, together with the fact that HCs have a unique phenotype, prompted an examination of the effect of αIFN on the expression of an extensive range of antigens on HCs. The results showed that αIFN caused profound effects on the phenotype of HCs; however, the combination of antigenic changes induced were difficult to interpret, but could be indicative of partial activation/differentiation. This work is reported in Chapter 1.

In order to establish whether the results of Chapter 1 were indicative of HC activation, or differentiation, an investigation of the effects of αIFN on the protein products of the proto-oncogenes *c-myc* and *c-fos* was undertaken. These molecules are involved in the signalling pathways for both activation (*c-myc* and *c-fos*) and differentiation (*c-fos*). Although αIFN did not effect the expression of the *c-myc* and *c-fos* proteins on unstimulated HCs, it caused down regulation of both in HCs that had been stimulated to proliferate. These results did not therefore shed any light on those reported in Chapter 1, but did indicate that αIFN acts in HCL by causing down regulation of the *c-myc* and *c-fos* proteins, the consequence of which is the cessation of HC proliferation. The details of these findings are reported in Chapter 2.

Although αIFN provides on anti-proliferative signal to HCs (Griffiths & Cawley 1991; Barut *et al* 1990; Chapter 2 of this Thesis) the nature of the *in vivo* proliferative signal to HCs was unknown. At the time work on Chapter 3 began it had been

recently reported that TNFα may provide this signal (Bianchi *et al* 1988; Cordingley *et al* 1988); since the cytokine both induces HC proliferation, and the secretion of TNFα by HCs. However as with other cytokines which had been reported to induce HC proliferation the levels of this proliferation were low. In addition, it was unclear as to whether TNFα was acting directly on the HCs; the cytokine could have acted on contaminating accessory cells, either to induce the proliferation of such cells or these cells could have been acting as accessory cells in the induction of HC proliferation. The effects of TNFα on highly purified HCs was therefore investigated. Since the cytokine had also been reported to induce the formation of TNFα independent HC lines, the effect of long term culture of HCs with the cytokine was also investigated. Proliferating HCs in these long term cultures formed close associations with macrophages; such associations are also seen *in vivo* and were shown to be responsible for the proliferation of HCs in a patient whose cells underwent proliferation in the absence of added growth factors (Griffiths & Cawley 1991). Since HCs *in vivo* also form association with other cells types, particularly endothelial cells (Re *et al* 1988), the specificity of this monocyte/macrophage effect was examined. Although many cell types were able to induce HC proliferation endothelial cells were the most potent. Investigation of the mechanisms responsible for this endothelial cell mediated HC proliferation indicated that both binding to endothelial cells via CD11b/CD18 and CD11c/CD18, and TNFα produced by the endothelial cells were necessary to induce maximum proliferation. The details of this work are contained in Chapter 3.

The results of previous chapters in this thesis emphasised the unique nature of the HC. One of the unusual features of HCs is their possession of antigens more commonly found on cells of other lineages, in particular monocytes. In cells of the monocyte lineage, a combination of the proto-oncogene *c-fms* and its ligand M-CSF are vital for the maintenance of the monocyte/macrophage state. This raised the possibility that the monocyte/macrophage features of HCs may be associated with expression of *c-fms*. Investigations showed that HCs did indeed express the *c-fms*

protein, and that binding of its ligand, M-CSF, stimulated HCs; this strengthened the suggestion that the monocytoid features of HCs could be explained by the receptor ligand combination of *c-fms* and M-CSF. However, although M-CSF has a number of effects on cells of the myeloid lineage, including the induction of proliferation, the cytokine was found not to influence any of these functions in HCs. The identification of *c-fms* proto-oncogene expression on HCs, and the investigation of the effects of M-CSF on HCs are reported in the fourth chapter of this thesis.

The remainder of this Introduction gives a brief background on the topics relevant to the work presented in this thesis viz. HCL, HCs, αIFN, the phenotype of HCs, oncogenes, B-cell growth and differentiation, *c-fms*, and M-CSF.

HCL

Clinical. HCL is a chronic lymphoproliferative disorder with a characteristic distribution of the leukaemic cells in the liver, spleen and bone marrow but not in the lymph nodes. HCs have a profound suppressive effect on the bone marrow, and death frequently occurs due to neutropenia (Reviewed in detail in Cawley *et al* 1980).

HCs. HCL was first described as leukaemic reticuloendotheliosis (Bouroncle *et al* 1958). At this time the cellular origin of HCs was unknown, although later work showed that they were activated B lymphocytes which possessed unique features (Debusscher *et al* 1975); these include the expression of surface antigens more commonly found on T cells (Worman *et al* 1984: Korsemeyer *et al* 1983) and monocytes (Braylan *et al* 1978; Debusscher *et al* 1975).

The activated B lymphocytes of HCL are more differentiated than the B cells of chronic lymphocytic leukaemia (CLL) and prolymphocytic leukaemia (PLL), but less so than the plasma cells of multiple myeloma (Foon *et al* 1986a). This fact is confirmed as both CLL and PLL cells can, when stimulated with phorbol esters, assume some characteristics of HCs (Zeigler-Hettbrock *et al* 1986); although HCs cannot be induced to differentiate into plasma cells (Griffiths & Cawley 1991).

In addition to these unique phenotypic features, HCs have a distinct morphology; as well as the characteristic projections which give them their hairy appearance (Borouncle *et al* 1958; Debusscher *et al* 1975), they have a distinctive ribosome lamellar complex (Debusscher *et al* 1975) and posses the tartrate resistant acid phosphatase (TRAP) enzyme (Foon *et al* 1986a). However, chromosomal abnormalities in HCL are rare.

αIFN

General. Binding of αIFN to its receptor induces internalisation and degradation of the receptor ligand complex (Evans *et al* 1984; Yonehara *et al* 1983), this results in a lasting down regulation of receptor numbers on the cell surface (Hannigan *et al* 1984). Internalisation of αIFN receptors is essential for the cytokine to exert its effects (Yonehara *et al* 1983); however, there is still controversy regarding the signalling steps occurring between internalisation and induction of the cellular response genes, such as the interferon response genes (ISG). αIFN treatment results in increased formation of diacyl glycerol (DAG) (Reich & Pfeffer 1990); this is not followed by turnover of phophatidyl inositol or increased Ca^{2+}, although the amount of the ε isoform of protein kinase C (PKC) does increase (Pfeffer *et al* 1991). The involvement of PKC in the signalling pathway of αIFN would correlate with the fact that the DAG analogue PMA, which activates PKC, induced phenotypic changes similar to those induced by αIFN on HCs (reported in Chapter 1). There is, however, controversy as to the role of PKC in αIFN signalling, as not all inhibitors of PKC inhibit ISG formation (Kessler & Levy 1991; Veals *et al* 1991). This has led to the speculation that more specific cellular kinases are involved in the signalling pathway of αIFN (Veals *et al* 1991), and that PKC is involved at the post rather that the pre-transcriptional stage (Kessler & Levy 1991). In addition to its effects on PKC, αIFN also causes changes in lipid metabolism resulting in increased concentrations of cAMP and cGMP (Clemens & McNurlan 1985; Pfeffer & Tan 1991), and the phosphorylation of a number of proteins (Clemens & McNurlan 1985; Pfeffer & Tan

1991; Yokoyama *et al* 1990). The various changes in signalling molecules which have been implicated following αIFN binding and internalisation are shown schematically in Fig. 1.

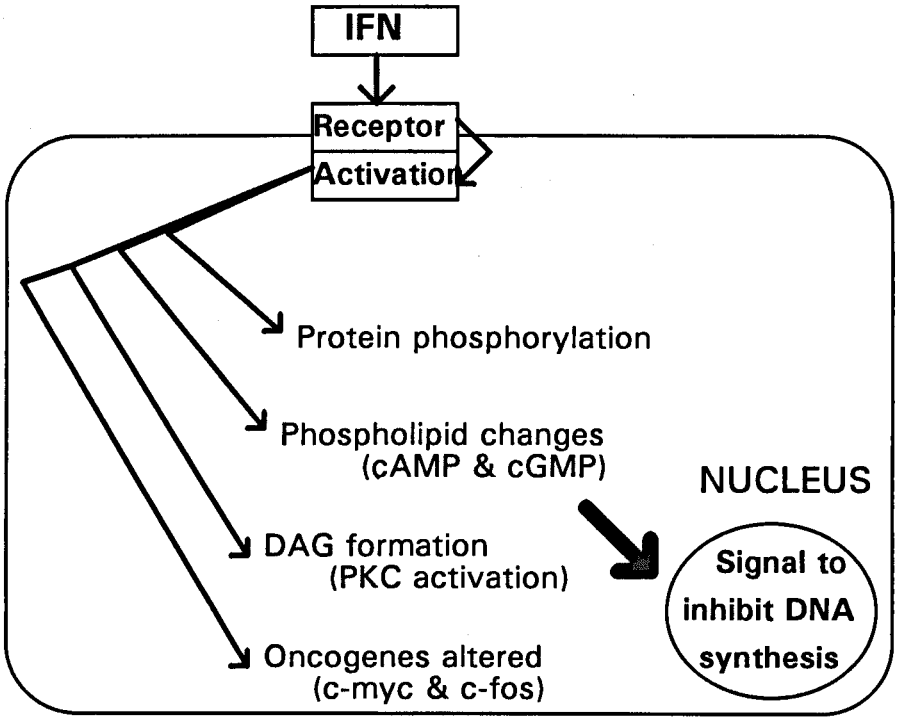


Fig. 1: Signalling pathway of αIFN

The effects of αIFN vary depending on the target cells; it can cause activation, differentiation, or, as also reported in this thesis, it can provide an anti-proliferative signal (Clemens *et al* 1985). αIFN exerts its anti-proliferative effect by stopping the passage of cells through the G0/G1 phase of the cells cycle (De Maeyer & De Maeyer-Guignard 1990; Lin *et al* 1986). This growth arrest in HCL is due, in part, to a decrease in the expression of the oncoproteins *c-myc* and *c-fos* (Chapter 2; Fig. 1). This anti-proliferative signal may be mediated by other cytokines; as MAbs to TGFβ block the actions of αIFN in a breast carcinoma cell line (Kerr *et al* 1989). In contrast to HCs, where αIFN is anti-proliferative, the cytokine provides a co-stimulatus to normal B cell proliferation in the presence of anti-μ (Morikawa *et al* 1987).

αIFN treatment of HCL. Since the first reported usage of αIFN in HCL (Quesada *et al* 1983) it has been the preferred treatment for HCL. However, more recently both deoxycoformycin (dCF) (Spiers *et al* 1984; Foon *et al* 1986b) and 2' chlorodeoxyadenosine (2'CDA) (Piro *et al* 1990; Juliusson *et al* 1992) have been shown to be more effective than αIFN in the treatment of HCL, and once clinical trials have been completed, they may replace αIFN as the preferred treatment for HCL. Splenectomy was historically used as the first line of treatment in HCL (Foon *et al* 1986a), and can give prolonged improvement in the disease. In the spleen HCs are found in close association with endothelial cells (Jansen *et al* 1984); this association results in HC proliferation (as discussed in Chapter 3); hence removal of the spleen would result in the loss of a site of tumour production, explaining the efficacy of this treatment.

Treatment regimes with αIFN vary, but all result in a slow improvement in the disease status in about 80% of patients (Thompson *et al* 1989; Federico *et al* 1989; Fland rin *et al* 1986). Treatment results in decreased HC numbers in the peripheral blood, spleen and bone marrow, and is accompanied by prolonged thrombocytopaenia (Ratain *et al* 1985; Berman *et al* 1990). The cytokine also causes an improvement in marrow function with a recovery in both erythropoiesis and granulopoiesis (Naem *et al* 1985; Hassan *et al* 1989). A decrease in marrow fibrosis is also seen in some patients in response to αIFN (Hassan *et al* 1989).

Both a lack of response (Berman *et al* 1990) and the loss of response (Besisz *et al* 1989) to αIFN has been attributed to the presence of antibodies to the cytokine (Steis *et al* 1991; Moormeier *et al* 1989). However, as is the case in one of the patients described in this study, resistance to αIFN is not always due to antibodies to the cytokine (Steis *et al* 1991). The majority of patients who are resistant to αIFN can now be successfully treated with dCF and 2' CDA (Spiers *et al* 1984; Piro *et al* 1990).

αIFN also alters the response of HCs to cytokines; it causes decreased proliferation in response to TNFα (Heslop *et al* 1990). Decreased proliferation of HCs in response to TNFα is seen to both recombinant TNFα, and TNFα produced by

endothelial cells; as reported in Chapter 3. αIFN also causes decreased proliferation in response to interleukin (IL) 6 and IL-2 (Heslop *et al* 1990).

αIFN treatment of other haematological malignancies. Since αIFN was first used in the treatment of HCL (Quesada *et al* 1983), the cytokine has been used with some degree of success in the treatment of other leukaemias. αIFN induces remission in about 75% of patients with chronic myelogenous leukaemia (CML) (Talpaz *et al* 1991; Schiffer *et al* 1991). In addition, the cytokine is used in the treatment of non-Hodgkins leukaemia (NHL) (Gresser 1991; Spiegel 1989), early stage CLL (Boussiotis & Pangalis 1991), and as maintenance therapy in multiple myeloma (Gresser 1991; Spiegel 1989). However, αIFN treatment is most successful in the treatment of HCL.

The phenotype of HCs

Antigenic profile of HCs. As already mentioned, the antigenic profile of HCs is unusual (Table 1). They posses antigens which are more commonly found on other lineages, e.g. CD25 (Robb *et al* 1981) and CD11c (Falini *et al* 1985); antigens characteristic of activated B cells, e.g. FMC7 (Bloem *et al* 1988) and HC2 (Visser *et al* 1989); and antigens which are found in higher levels on HCs than other B lymphocytes, e.g. CD22 and HLA DR (Schwarting *et al* 1985). The effects of αIFN on the surface antigen expression of HCs were therefore investigated. The antigens studied were chosen either because they were markers of HCL CD25, CD11c, CD22, HC2 and FMC7; altered in B cells on activation CD23, CD71 and MHC class II or differentiation, CD38; or they were B-cell markers found on HCs, CD9, CD19, CD20, CD24, CD39 and CD40. Since this study was completed both the range of CD antigens and antigens which are markers for HCL has increased.

HC antigens involved in activation and differentiation. Many antigens found on HCs are either associated with or intimately involved in the activation/differentiation of B lymphocytes. Those which have well defined functions, and are examined in this thesis, are the CD19, CD20, CD22, CD23, CD25 and CD40 antigens.

Antigen	Expression	Antigen	Expression
CD1a	+	CD36	-/+
CD2	-/+	CD37	+
CD9	++	CD39	+
CD11a	-/+	CD40	++
CD11b	+	CD43	+
CD11c	++	CD44	++
CD18	++	CD49	-/+
CD19	++	CD54	++
CD20	++	CD74	++
CD22	++	CD75	++
CD23	-/+	CD76	++
CD24	+	CD78	+
CD25	+	B-ly7	++
CD26	++	HC2	++
CD29	+	FMC7	++
CD31	+	Class I	++
CDw32	++	Class II	++

Table 1: Antigens expressed by HCs. -/+, variable; +, weak to moderate; ++, strong. (Adapted from Cawley & Burthem 1991 with permission.)

The CD19 antigen is found on all stages of the B lymphocyte lineage from pre-B cells onwards, but is lost on terminal differentiation of these cells to plasma cells (De Rie *et al* 1989). The CD19 molecule is involved in the regulation of B lymphocyte responses, with MAbs to this antigen inhibiting both activation (Callard *et al* 1992; Golay & Crawford 1987; Pezzutto *et al* 1988b) and differentiation (Tedder *et al* 1985: Lewis *et al* 1987). It is also associated with surface immunoglobulin (sIg); capping of sIg causes decreased CD19 expression (Pesando *et al* 1987). In HCs treated with αIFN, both CD19 and sIg expression are reduced (Chapter 1).

The CD20, CD22 and CD40 molecules are all involved in B lymphocyte activation; MAbs to these molecules provide a co-stimulus for B lymphocytes to enter the cell cycle (Clark & Ledbetter 1986b; 1987; Gordon *et al* 1987a; Pezzutto *et al*

1987a; Wilson *et al* 1991). MAbs to CD20 also inhibit B lymphocyte differentiation (Golay *et al* 1985). In addition, the CD22 (Stamenkovic & Seed 1992) and CD40 (Armitage *et al* 1992) molecules are also involved in the adhesion of B lymphocytes.

Both the CD23 and CD25 molecules are receptors; CD23 is the low affinity receptor for IgE (Bonnefoy *et al* 1987; 1988) and the CD25 antigen is the low molecular weight receptor for IL-2 (Jung *et al* 1984). MAbs to CD23 stimulate B lymphocytes in a similar way to B-cell growth factor (BCGF) (Gordon & Guy 1987b; 1986).

The major histocompatibility (MHC) class II molecules are also involved in B lymphocyte activation; the expression of these molecules is increased on activation of both B lymphocytes (Mond *et al* 1981; Godal *et al* 1985) and HCs (Gamliel *et al* 1990; Griffiths & Cawley 1991). Higher levels of class II antigen expression are associated with increased capacity of B lymphocytes to present antigen to T-cells (Van Heyningen *et al* 1982; Gonowa *et al* 1983).

Change in HCs antigens on stimulation. Treatment of HCs with mitogens which induce their proliferation causes phenotypic changes. Stimulation of HCs with both BCGF and TNFα causes increased expression of CD25 and HLA DR (Gamliel *et al* 1990; Griffiths & Cawley 1991; Chapter 3). However, since HCs cannot be induced to fully differentiate into plasma cells (Griffiths & Cawley 1991) the antigenic changes induced on HC differentiation remain unknown.

Regulation of antigen expression by αIFN. The expression of MHC class I antigens is increased by αIFN (Nissen *et al* 1987; Burrone & Milstein 1982 Erualimsky *et al* 1987). Whereas αIFN is involved in the regulation of MHC class I antigen expression, γIFN is involved in the regulation of MHC class II molecules, both inducing (Mauer *et al* 1987; Collins *et al* 1984) and up regulating (Rosa *et al* 1986) their expression. In contrast to most other cell systems, αIFN causes both the expected increase in class I and increased DR (class II) MHC on HCs (Baldini *et al* 1986: Gamliel *et al* 1988). These *in vitro* changes in HLA are also seen on HCs following *in vivo* treatment of HCL (Huang *et al* 1987).

αIFN also causes altered expression of a number of other surface molecules. The cytokine causes an increase in the levels of the intercellular adhesion molecule ICAM-1 (CD54) on melanoma cells (Maio *et al* 1989). It inhibits the expression of the transferrin receptor (Besancon *et al* 1987). αIFN also effects the expression of Fc receptors on lymphocytes; on T cells it increases the expression of Fcγ (Fridman *et al* 1980), while suppressing the expression of FCμ receptors (Itoh *et al* 1980). αIFN also induces the expression of CD69 on NK cells (Gerosa *et al* 1992). αIFN treatment also causes a general increase in HC protein synthesis (Samuels *et al* 1987a; 1987b; Cellis *et al* 1987; Beresini *et al* 1988). In addition the cyto kine effects the release of cell surface proteins from HCs resulting in increased levels of sCD23 (Genot *et al* 1989) and sCD8 (Ho *et al* 1990).

Oncogenes

General. Proto-oncogenes are important in the process of neoplasia, although they encode proteins which are involved in the day to day running of the cells (Fig. 2) they can undergo transformation to become oncogenes. Transformation of oncogenes occurs in a variety of ways (Table 2), however, all result in abnormal growth kinetics in the affected cells. A number of such transformations have been shown to be involved in lymphoid malignancies (Table 2). Only three of these oncogenes are both regulated by αIFN and involved in the signalling pathways of normal cells; *c-myc, c-fos* and *ras* (Jonak & Knight 1986; Verma *et al* 1986; Erandi *et al* 1987; Barizilay *et al* 1987; Golay *et al* 1990). Regulation of these oncogenes may therefore be an important component of the mechanism by which αIFN exerts its effects; however of these three proto-oncogenes HCs only express *c-myc* and *c-fos* (Giron *et al* 1989; Lehn *et al* 1989). An investigation of the effects of αIFN on the expression of these two oncogenes was undertaken, and this is reported in Chapter 2 of this thesis.

C-fos. The *c-fos* protein is located in the nucleus (Verma *et al* 1986), and it regulates transcription by binding to specific sites on DNA. *C-fos* expression is elevated in normal B cells following differentiation induced by both PMA and αIFN

(Erandi *et al* 1987), the proto-oncogene also causes differentiation when transfected into embryonal carcinoma cells (Ruther *et al* 1985). *C-fos* is also elevated in response to growth stimuli by mitogens both in haematopoietic cells and fibroblasts (Muller *et al* 1984; Reed *et al* 1986). Since HCs are differentiated B lymphocytes they, express higher amounts of *c-fos* than other B-cell leukaemias (Pinto *et al* 1987). High levels of *c-fos* expression are also seen in myeloid leukaemias of the differentiated, M4 and M5 types (Mavilo *et al* 1987). In B cells *c-fos* is a component of the membrane immunoglobulin signal transduction mechanism (Munroe *et al* 1988), being involved in the transition from the G0 to the G1 phases of the cell cycle. It is therefore an early component in the activation of B cells, but its presence is also necessary for passage through the later stages of the cell cycle.

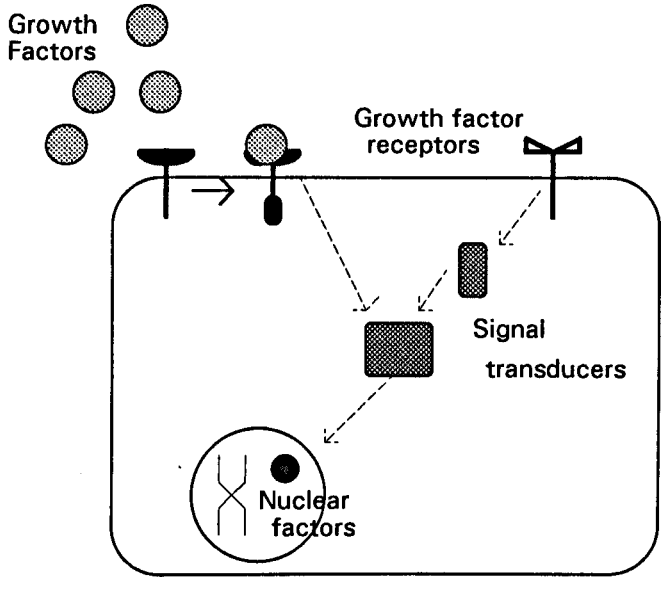


Fig. 2: Schematic representation of the functions ascribed to proto-oncogenes.

Increased expression of *c-fos* is also related to increased expression of the MHC antigens. An increase in the mRNA coding for *c-fos* occurs 1-4 hours after PMA is added to lymphoma cells, this is followed by an increase in the mRNA coding for MHC antigens which is maximal 12 to 24 hours after stimulation (Barizilay *et al* 1987). In mouse 3LL cells αIFN causes *de novo* transcription of mRNA coding for

both *c-fos* and MHC antigens (Kushtai *et al* 1987), with the *c-fos* protein expression inducing MHC antigen expression.

Onc.[a]	Normal function	Activation	Disease
abl	Tyrosine kinase Signal transducer	Translocation→Hybrid gene (*bcr*) Amplification	CML
bcr	Phosphoprotein	Translocation→Hybrid gene (*abl*)	CML
fos	Nuclear protein	NK[a]	
fms	Tyrosine kinase Growth factor receptor (M-CSF)	Single base substitution (Mutation)	AML
ras	GTP binding/GTPase Signal transducer	Single base substitution (Mutation) Amplification	ALL AML MDS
kit	Tyrosine kinase Growth factor receptor (SCF[b])	NK	
myb	Nuclear factor	Amplification	CML AML ALL
myc	RNA processing	Translocation Amplification	BL[c] PML
sis	Growth factor (PDGF agonist)	? Translocation	AML

Table 2: Normal functions of oncogenes involved in haematopoiesis, and their mechanism of activation in lymphoid malignancies. a) NK=no known involvement in lymphoid malignancies. b) SCF=stem cell factor. c)BL=Burkitts lymphoma. (Westin *et al* 1982; Mavillo *et al* 1986; Blick *et al* 1984; McKenzie 1991; Billips *et al* 1992)

C-myc. Although *c-myc* has been extensively studied the function of this proto-oncogene is not completely understood. It may encode for a transcription factor (Golay *et al* 1992), and hence be an important regulator of transcription (McKenzie 1991). Increased expression of *c-myc* precedes the incorporation of both tritiated uridine and thymidine on cell division, and is necessary for cell division to occur (Golay *et al* 1992). In contrast to its role in growth, *c-myc* expression is decreased on cellular differentiation; in fact elevated levels of this proto-oncogene prevent differentiation (Dang 1991). As would be expected of highly differentiated cells with a slow growth rate, HCs express negligible amounts of *c-myc* (Lehn *et al* 1986).

Effects of αIFN. In addition to causing increased expression of *c-fos*, resulting in increased MHC antigen expression, αIFN also causes decreased *c-fos* expression in both Rous sarcoma virus infected rat cells (Fridman 1980) and fibroblasts (Jonak & Knight 1986; Yaar *et al* 1990). αIFN treatment causes decreased *c-myc* expression in both haemopoietic cells and fibroblasts (Resnitzky *et al* 1986; Blondel *et al* 1991), and in the uniquely sensitive Burkitts lymphoma cell line (Jonak & Knight 1984; Meadows *et al* 1990) operating at the post-transcriptional level (Knight *et al* 1985). This decreased *c-myc* expression causes growth arrest in these cells (Einat *et al* 1985) at the G0/G1 interphase (Resnitzky *et al* 1986). αIFN acts on *c-myc* and *c-fos* gene expression at the post-transcriptional stage (Levine *et al* 1990).

B-cell growth and differentiation

Cytokines. A number of cytokines are involved in the growth and differentiation of B lymphocytes (Table 3). However, this thesis concentrates on the effects of one of these cytokines, TNFα, on HCs. Therefore this section of the introduction will concentrate on this cytokine. The effects of other cytokines on B lymphocytes and HCs is reviewed extensively elsewhere (Thomas 1992; Griffiths 1990).

Cytokine	Production	B-cell actions	Effect on HCs	
			Prolif.[a]	Diffn.[b]
IL-1	Lymphoid Stromal cells	Synergy in differentiation Synergy in growth	-	ND[c]
IL-2	T lymphocytes Monocytes	Growth - activated B cells Differentiation - activated B cells	-	-
IL-3	T lymphocytes	Differentiation - pre-B cells	ND	ND
IL-4	T lymphocytes Mast cells	Co-stimulates proliferation Differentiation	-	-
IL-5	T lymphocytes	None (in humans)	ND	ND
IL-6	T lymphocytes Stromal cells	Differentiation - activated B cells	-	-
IL-7	Stromal cells	Proliferation - pro- & pre-B cells	ND	ND
TNFα	Monocytes Stromal cells	Proliferation - activated B cells	+	-
SCF[d]	Stromal cells	Co-stimulates growth -pre-B cells	ND	ND

Table 3: Cytokines affecting B lymphocytes, and their effects on HCs. a) Prolif.=proliferation. b) Diffn.=differentiation. c) ND=not done. d) SCF=stem cell factor (Griffiths & Cawley 1991; Thomas 1992; Nakagawa *et al* 1987; Miyawki *et al* 1987; Palacious 1984; Snapper *et al* 1988; Lebman & Coffman 1988; Matsumoto *et al* 1989; Sanderson *et al* 1988; Aarden & Van Kooten 1992; Roldan & Breva 1991; Billips *et al* 1992; Hayashi *et al* 1991).

TNFα can be either membrane bound (Kreigler *et al* 1988) or secreted (Aggarwal *et al* 1985). The secreted form is a disulphide bonded glycoprotein with a molecular weight of 17kD (Aggarwal *et al* 1985), which is cleaved from the 26kD

membrane bound form (Kreigler *et al* 1988). TNFα is mainly produced by macrophages (Penicca *et al* 1988; Debetts *et al* 1988), but is also produced by both stimulated normal B cells (Sung *et al* 1988). and by the leukaemic B cells of HCL and CLL (Foa *et al* 1990).

TNFα is involved in cell killing by both monocytes (Feinman *et al* 1987) and macrophages (Decker *et al* 1987). The cytokine also inhibits the *in vitro* growth of both normal (Moore *et al* 1985) and leukaemic (Broxmeyer *et al* 1986) haemopoietic progenitor cells. This suppression is not due to the cytotoxic effects of TNFα (Murase *et al* 1987), and is thought to contribute to the haemopoietic failure seen in HCL (Lindermann *et al* 1989).

Although TNFα was originally described as a cytotoxic molecule it also has stimulatory effects. The cytokine induces the differentiation of both HL60 cells (Hemmi *et al* 1987) and a myeloid leukaemia cell line, M1 (Onozaki *et al* 1988). It also induces the production of a number of cytokines, including IL-1 (Locksley *et al* 1987) and GM-CSF (Munker *et al* 1986). TNFα also enhances the differentiation of B cells induced by poke weed mitogen (PWM) (Jelenek & Lipsky 1987). As well as its differentiating effect on B cells, TNFα is involved in their growth. The cytokine induces the proliferation of leukaemic B cells from both CLL (Digel *et al* 1989; Bianchi *et al* 1988) and HCL (Cordingley *et al* 1988; Buck *et al* 1990). This proliferation can be stimulated by both secreted and membrane bound TNFα (Hoesli & Nissen-Meyer 1989).

The effects of TNFα on the proliferation of HCs are investigated further in the first part of Chapter 3.

Stromal cells. The growth and differentiation of haemopoietic cells both *in vivo* and *in vitro* is directed by accessory cells of the bone marrow stroma (Whitlock & Witte 1982) and lymphoid organs (Kosco *et al* 1992). Lymphopoiesis in long term cultures is dependent on an adherent layer (Dorshkind & Witte 1987). These adherent cells comprise a mixed population of fibroblasts and macrophages (Wolf *et al* 1991); although epithelial cells in the foetal liver (Dorshkind & Witte 1987) and in the avian

Bursa of Fabricius (Reynolds 1987) also support the growth and differentiation of B lymphocytes. Both adherence to and growth factors produced by these adherent stromal cells are essential for the growth and differentiation of B lymphocytes in long term cultures.

A number of growth factors are produced by stromal cells (Table 3). The proliferation of pre- and pro-B lymphocytes is mediated by IL-7 (Takai *et al* 1992; Saeland *et al* 1991); with the effects of this cytokine being augmented by IL-3 (Saeland *et al* 1991) and stem cell factor (Funk & Witte 1992). Whereas the differentiation of more committed B cells in these cultures is mediated by IL-6 (Roldan *et al* 1992), IL-6 is also responsible for the differentiation of B lymphocytes in lymphoid tissue where it is produced by follicular dendritic cells (Kosco *et al* 1992).

Lymphocytes in long term cultures adhere to both the stromal cells themselves and to the extracellular matrix (ECM) components produced by these cells. The binding of the lymphocyte homing receptor CD44 to its counter-receptor on the stromal cells in long term bone marrow cultures (LTBMC) is essential for the production of lymphocytes (Mikaye *et al* 1990). In addition binding to fibronectin of the ECM is also important; antibodies to this protein markedly inhibit the proliferation of pre-B lymphocytes (Lemoine *et al* 1990) and the differentiation of B-lymphocytes (Roldan *et al* 1992) in LTBMC. The differentiation of B lymphocytes in the lymphoid organs, in contrast, is dependent on cell-cell adhesion via CD11a binding to CD54 (Kosco *et al* 1992). However, this adhesion of lymphocytes to stromal cells/ECM components does not itself mediate the proliferation/differentiation of B lymphocytes, it acts in synergy with cytokines produced by these stromal cells (Lemoine *et al* 1990).

Association of HCs with accessory cells *in vivo*. HCs infiltrate the red pulp of the spleen (Jansen *et al* 1984) causing atrophy of the peri-arteriolar lymphatic sheath (PALS) (Nanba *et al* 1977). As a result of this infiltration the number of macrophages in the PALS decreases, and this is accompanied by an increase in the numbers of both macrophages and histocytes in the cord area (Nanba *et al* 1977). The follicular and

central arteries also degenerate and atrophy (Nanba *et al* 1977). In the PALS HCs form pseudosinuses (Re *et al* 1988), comprising up to 95% of the sinuses in the HCL patients spleen. HCs therefore totally destroy the normal architecture of the spleen.

HCs also infiltrate the bone marrow, where they form between 30 and 95 % of all marrow cells (Coci *et al* 1987). Infiltration results in both a decrease in normal haematopoiesis, and an increase in the reticulin fibres resulting in marrow fibrosis (Coci *et al* 1987). This marrow fibrosis is due to HCs themselves producing fibronectin (Burthem & Cawley 1993), whereas the decrease in haematopoiesis is thought to be partly due to the production by HCs of high levels of TNFα (Linderman *et al* 1989).

The role of accessory cells in HC proliferation and the mechanisms responsible for the observed proliferation are examined in the second part of Chapter 3.

C-fms

Characterisation. The *c-fms* proto-oncogene is derived from the Susan McDonough feline sarcoma virus *v-fms* (McDonough *et al* 1987); with the *v-fms* protein having an extra 29 amino acids at its carboxy terminal (Sherr & Rettenmier 1986), and a different ligand binding domain (Rohrschneider & Metcalf 1989). The *c-fms* protein is the receptor for the macrophage colony stimulating factor (M-CSF), which is also known as colony stimulating factor-1 (CSF-1) (Sherr *et al* 1985). The receptor is a transmembrane glycoprotein with a molecular weight of 150kD (Sherr 1990; Rettenmier *et al* 1988), the genes for which are located on the short arm of chromosome 5 (Rettenmier & Sherr 1990).

Transforming potential. *C-fms* transformation occurs due to specific point mutations. These mutations map both within (Rettenmier 1989) and outside the M-CSF binding domain (Roussel *et al* 1988). Mutations of *c-fms* lower the affinity of the receptor for its ligand, and cause it to be consitutively phosphorylated on tyrosine (Roussel *et al* 1988). The mutated *c-fms* is not regulated by binding of M-CSF (Sherr 1988), and therefore signals to the cell in the absence of ligand.

Expression. *C-fms* expression among normal haemopoietic cells is limited to those of the myeloid lineage (Sherr *et al* 1985). The amount of *c-fms* expressed by cells of the monocyte lineage increases as the cells become more differentiated (Liebermann & Hoffman-Liebermann 1989; Radzun *et al* 1988); the highest expression is therefore found on macrophages (Sherr *et al* 1985). Treatment of the myelomonocytic cell line HL60 with PMA, which induces its differentiation along the monocyte pathway, induces *c-fms* (Rettenmier *et al* 1986; Wu *et al* 1990; Sariban *et al* 1985; Neinhuis *et al* 1985). Transfection of *c-fms* into cells of other lineages also induces myeloid differentiation in response to M-CSF; on removal of the cytokine the transfected cells revert to their original features (Rohrschneider & Metcalf 1989). Therefore, the combination of receptor (*c-fms*) and ligand (M-CSF) is important in the induction and maintenance of the myeloid state.

C-fms expression is also found on myeloid leukaemias (Tamura *et al* 1989), where its expression by AML cells, as with monocytes, is related to their stage of monocytic differentiation (Parawesch *et al* 1990); with the highest expression being found among the highly differentiated M5 group (Dubriel *et al* 1988; Ashmun *et al* 1989). However, AML blasts, in the main, do not express *c-fms* (Ashmun *et al* 1989). *C-fms* expression by malignant haemic cells may not be confined to those of the myeloid lineage; expression of the proto-oncogene has been described on a Hodgkins lymphoma derived cell line (Paietta *et al* 1990).

Regulation. Expression of the *c-fms* protein is regulated by its ligand M-CSF, with binding of the cytokine inducing down regulation of its receptor (Sherr *et al* 1988; Sariban *et al* 1989). This down regulation of the *c-fms* protein is a result of the internalisation and degradation of the receptor following binding of M-CSF (Li *et al* 1991; Guilbert & Stanley 1986; Carlberg *et al* 1991). *C-fms* expression is also regulated by other cytokines, with TNFα (Sheih *et al* 1989) and PMA (Downing *et al* 1989) causing decreased expression; while GM-CSF has no effect (Espinoza-Delgado *et al* 1990).

Signal transduction. *C-fms* has ligand activated tyrosine kinase activity (Varticovski *et al* 1989; Sherr 1988). Binding of M-CSF causes rapid phosphorylation, on four different sites, of the *c-fms* protein on tyrosine (Sengupta *et al* 1988; Downing *et al* 1988; Tapley *et al* 1990). Following phosphorylation of the *c-fms* protein, a number of other proteins are phosphorylated (Yeung *et al* 1987; Van Der Gerr & Hunter 1990). Activation of *c-fms* induces the cellular response to M-CSF. Phosphorylation of *c-fms* induces phosphorylation of inositol 3' phosphate kinases (Reedijk *et al* 1990; Sherr *et al* 1990) together with G protein activation, which in turn induces PKC activation via phospholipase C (Imamura *et al* 1990; Choudhury *et al* 1991; Vairio *et al* 1991). Phosphorylation of the *c-fms* protein also induces Na^+H^+ and Na^+K^+ ion exchange and the uptake of glucose by the cell (Imamura *et al* 1990; Vario *et al* 1990; 1991).

The expression of the *c-fms* protein by HCs is discussed in the first part of Chapter 4.

M-CSF

Characterisation. M-CSF exists in multiple forms, it can be either membrane bound (Rettenmier *et al* 1987) or soluble (Stein *et al* 1990); with the soluble form being derived from cleavage of membrane M-CSF (Stein *et al* 1990). The molecular weight of M-CSF varies around 70kD (Tushinski *et al* 1982); this heterogeneity is due to both pre- and post-transcriptional processing (Shadle *et al* 1989). At the pre-transcriptional level differential splicing of mRNA is responsible for this heterogeneity (Ladner *et al* 1987; Stein *et al* 1990), whereas differences in glycosylation are responsible for the post-transcriptional modifications (Shadle *et al* 1989). Secreted M-CSF is always in the form of a disulphide linked heterodimer (Rettenmier & Sherr 1990; Metcalf 1990).

Production. M-CSF is produced by activated lymphocytes of all lineages. Resting monocytes are unable to produce M-CSF (Rambaldi *et al* 1987); however, activation by a variety of stimuli, including γIFN, TNFα and PMA, induces

production of M-CSF (Horiguchi *et al* 1986; Rambaldi *et al* 1987; Sherman *et al* 1990). Macrophages are also able to produce M-CSF on stimulation (Sherr 1990). Similarly, activation of both T (Ceridan *et al* 1990) and B lymphocytes (Reisbach *et al* 1989) induces M-CSF secretion. Leukaemic cells of all lineages have been shown to constitutively secrete M-CSF (Nakamura *et al* 1989; Reisbach *et al* 1989; Hallet *et al* 1991).

M-CSF is also produced by cells which are involved in the regulation of haematopoiesis. Stromal cells of the bone marrow produce M-CSF constitutively (Rettenmeir & Sherr 1990; Rambaldi *et al* 1987). Fibroblasts also produce the cytokine in the absence of stimulation, whereas endothelial cells require stimulation in order to produce M-CSF (Rettenmier & Sherr 1990; Sherr 1990).

Cellular effects. The results of M-CSF stimulation on cells of the monocyte lineage are numerous; it is involved in their proliferation, differentiation, survival, morphology, phenotype and cytotoxicity (Sariban *et al* 1989; Mufson *et al* 1989).

The differentiation of cells of the myeloid lineage in response to M-CSF is seen both *in vivo* and *in vitro* (Nakoinz *et al* 1990; Ulich *et al* 1990; Hume *et al* 1988). M-CSF causes differentiation throughout the myeloid lineage; from very primitive myeloid precursors (Sherr *et al* 1988) to monocytes (Yeung *et al* 1990). The differentiating activity of M-CSF is often accompanied by proliferation (Pierce *et al* 1990), and results in phenotypic changes (Young *et al* 1990). *In vivo* administration of M-CSF causes an increase in haematopoiesis, resulting in the production of mature tissue macrophages in the spleen (Hume *et al* 1988), bone marrow (Ulich *et al* 1990), and liver (Bock *et al* 1991).

M-CSF also causes proliferation of myeloid cells both *in vivo* (Ulich *et al* 1990; Hume *et al* 1988) and *in vitro* (Tushinski & Stanley 1985). The cytokine induces the proliferation of myeloid cells at all stages of differentiation (Tushinski & Stanley 1985). Although M-CSF induces the proliferation of monocytes on its own, its proliferative potential is increased in the presence of other cytokines, including TNFα (Chen & Meuller 1990) and IL-6 (Bot *et al* 1989).

Levels of M-CSF which are too low to induce monocyte/macrophage proliferation induce the long term survival of these cells (Tushinski & Stanley 1985; Brugger *et al* 1991); this increased survival is due to suppression of apoptosis (Williams *et al* 1990).

M-CSF also induces changes in the morphology and phenotype of myeloid cells. The cytokine causes an increase in the vacuolation, membrane ruffling and the number of filopodia on macrophages; these changes are accompanied by an increase in macrophage size (Tushinski *et al* 1982). The phenotype of macrophages produced in response to M-CSF is different from those seen with other cytokines (Breen *et al* 1991); M-CSF induced macrophages have more CD14 and CD16 (Young *et al* 1990; Geisler *et al* 1989). M-CSF also causes reduced antigen expression on macrophages (Willman *et al* 1989); the cytokine decreases the amount of MHC class II molecules.

M-CSF enhances the killing capacity of both monocytes (Khwaja *et al* 1991) and macrophages (Young *et al* 1990; Munn & Cheung 1989). This increase in macrophage killing is due to both an enhancement of their ability to mediate antibody dependent cellular cytotoxicity (Mufson *et al* 1989), and an increase in the secretion of TNFα (Sampson-Jonannes *et al* 1988; Bock *et al* 1991). M-CSF also affects the oncogene expression of myeloid cells, causing increases in *c-myc*, *c-fos* (Schaberg & Filderman 1992), *c-raf* (Choudhury *et al* 1992), *c-jun* and *hck* (Mufson *et al* 1990). In addition, M-CSF causes the production of fibronectin by monocytes (Mufson *et al* 1990) and increases their chemotaxis and chemokinesis (Kwaja *et al* 1991).

Although no effects of M-CSF on HCs are reported in Chapter 4, work performed in this Department has subsequently shown that M-CSF alters the motility and adhesive properties of HCs (Burthem *et al* 1993). Work is also underway to establish whether HCs in common with other activated B lymphocytes synthesise M-CSF. If this is indeed the case, in addition to its effects on HC locomotion autocrine M-CSF may be involved in the maintenance of the monocytoid features of HCs, as is seen in cells of the myeloid lineage.

THE EFFECTS OF αIFN ON THE SURFACE ANTIGEN EXPRESSION OF HCS

CHAPTER 1

INTRODUCTION

HCs express a unique combination of proteins on their cell surface. Although HCL results from clonal expansion of B cells (Korsemeyer *et al* 1984), HCs express antigens which are found predominantly on cells of both the T lymphocyte (CD25, CD2) (Bentaboulet *et al* 1987; Worman *et al* 1982) and macrophage lineages (CD11c) (Schwarting *et al* 1985). The expression of other antigens, for example B-ly 7 (Thaler *et al* 1990; Mulligan *et al* 1990) and HC2 (Posnett *et al* 1982; Posnett & Marboe 1984), is largely confined to HCs. As is to be expected, HCs also express antigens found on normal activated B cells, for example FMC7 (Ferro and Zola 1990), CD22 (Visser *et al* 1989), and high levels of HLA DR (Baldini *et al* 1986).

The cytokine αIFN alters the protein synthesis of cells to which it is administered (Cellis *et al* 1987; Beresini *et al* 1988). Treatment of HCs with αIFN *in vitro* induces the expression of a range of proteins that are not found on other cells following treatment with the cytokine (Samuels *et al* 1987a; 1987b). In addition, at the time work on this chapter began, a study examining the effects of αIFN on the expression of a small number of HC surface antigens had recently shown that the cytokine increased the expression of MHC molecules (Baldini *et al* 1986).

The aim of this chapter was to investigate further the effects of αIFN on a wide range of antigens expressed on the surface of HCs. It was hoped that the changes observed as a result of αIFN treatment of HCs *in vitro* would shed some light on the mechanism(s) of its action *in vivo.*

PATIENTS AND METHODS

Patients

HCs were studied from 6 patients with HCL; all had typical disease, although one was unusual both in that HCs from this patient both underwent proliferation *in vitro* in the absence of added growth factors and were refractory to αIFN treatment *in*

vivo. Peripheral blood (PB) HCs were examined in all cases, and in 2 cases splenic HCs were also examined.

PB was also examined from 4 cases of CLL; 2 had stage IV, while one each had stage 0 and II disease.

Buffy coat cells (Blood Transfusion Service) were used as a source of normal B cells.

Cell preparation

Peripheral blood mononuclear cells (PBM) were isolated by density gradient sedimentation over lymphoprep. Where T lymphocytes formed >5% of the cell population they were removed as follows; PBM were incubated at 4°C for 1 hour with 2-aminoethylisothiouronium bromide hydrochloride (AET) treated sheep red blood cells (SRBC). (Under these conditions T cells form rosettes with SRBC.) T cells were then removed by density gradient centrifugation over lymphoprep. Where monocytes formed >5% of the resulting PBM they were removed by incubating the cells on petri dishes for 1 hour at 37°C and removing the non-adherent cells. This resulted in >95% CD19 positive B cells (CD3<5, CD14<5%).

Slices of spleen were collected from theatre into bottles of RPMI containing 10U/ml of heparin. The spleen was then cut into 0.5cm^3 pieces which were placed separately between 2 pieces of fine wire mesh in a petri dish containing RPMI. The tissue was then dispersed by pressing down on the wire mesh using a syringe plunger. Cells dispersed in this manner were then pipetted off and, after allowing small pieces of tissue to settle out, HCs were separated from the cell suspension by density gradient sedimentation over lymphoprep. HCs obtained this way were >90% viable and >98% pure.

All cells were frozen in liquid nitrogen prior to use. Briefly, cells were suspended in 50% autologous plasma in RPMI and 10% dimethylsulphoxide (DMSO) was added. 1ml aliquots were then insulated and frozen at -70°C overnight before

transferring to liquid nitrogen. In one case, however, both frozen and fresh HCs were used and comparable results obtained (data not shown).

Serum	αIFN (U/ml)	2 days mode x 10^{5a}	%	4 days mode x 10^{5a}	%
FCS	0	0.8	95	1.1	97
	100	30	98	0.6	92
AB	0	0.4	92	1.0	90
	100	40	99	12	99

Table 1: The effects of FCS and AB serum on the αIFN induced increase in DR. a) See p.28 for calculation of results.

Cells were cultured in RPMI containing 10% human AB serum, 100μg/ml streptomycin, 100U/ml penicillin and 2mM L-glutamine (ICN-Flow). AB serum was used in preference to foetal calf serum (FCS) as FCS was found to partially inhibit the increase in HLA DR produced by αIFN (Table 1). Cells were cultured either in the presence or absence of 100U/ml αIFN (Wellcome). The dose of αIFN was chosen following titration against the increase it produced in HLA DR (Table 2), and because it is within the range of plasma concentrations found in treated HCL patients. HCs were also cultured in the presence or absence of 100U/ml γIFN (Roche) or 100ng/ml PMA (Sigma).

αIFN (U/ml)	Mode x 10^{5a}	%
0	0.8	93
20	12	99
100	14	96
500	8.8	92

Table 2: Titration of αIFN against the increase in HLA DR at 2 days. a) See p.28 for calculation of results.

Cells were cultured at a concentration of 2 x 10^6/ml in 24 well plates (Falcon) for up to 8 days at 37°C in 5% CO_2 in air and sampled every 2 days for FACS analysis. In one experiment (2 patients) cells were cultured, as above, and harvested at 2 and 4 hours, and then every 4 hours up to 24 hours.

Staining

MAbs were used against the following antigens: CD3 (Leu 4), CD4 (Leu 3a), CD5 (Leu 1), CD9 (BA2), CD10 (CALLA), CD11b (Leu 15), CD11c (Leu M5), CD14 (Leu M3), CD16 (Leu 11a), CD18 (a gift from Dr. Teteroo), CD19 (Leu 12), CD20 (Leu 16), CD21 (CR-2), CD22 (Leu 14), CD23, (MHM6, a gift from Prof. McMichael), CD24 (BA-2), CD25 (Tac), CD37 (WR17), CD38 (HB7, a gift from Dr. Ledder), CD39 (G28-10, a gift from Dr. Leadbetter), CD71 (transferrin receptor) DP, DQ (Leu 10), DR, class II MHC (WR18, a gift from Dr. Smith), class I MHC (W6/32), κ, λ, HC2 (a gift from Dr. Posnett) and FMC7.

Prior to staining by indirect immunofluorescence, cells were incubated at 37°C for 30 minutes in RPMI containing 15% FCS to remove cytophylic immunoglobulin. The cells were washed and incubated in microtitre plates for 20 minutes at 4°C with saturating concentrations of MAbs (2 x 10^5 cells/well), and then washed twice in phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 0.1% azide (PBS/BSA/azide). The cells were then incubated for 20 minutes at 4°C with 10% FITC-labelled goat anti-mouse immunoglobulin in PBS/BSA/azide containing 1% human AB serum, and washed a further 2 times in PBS/BSA/azide. The stained cells were then kept at 4°C until analysed. Non-immune IgG1, IgG2a, IgG2b, IgG3 and IgM were used as class specific controls.

FACS analysis

Cells were analysed on a FACS analyser I, with consort 30 computer and software (Becton Dickinson). 5,000 cells were analysed for each MAb and class specific control. For each MAb the percentage of positive cells, the modal

fluorescence and the volume were measured. The modal fluorescence is proportional to the number of antigen sites per cell for each individual MAb.

Calculation of results

The modal fluorescence was converted into the number of molecules of FITC per cell, and the cell volume was calculated using a calibration line drawn using beads of a known fluorescence and volume.

The percentage difference between treated and control cells for modal fluorescence was calculated using the following formula:

$$\text{Percentage difference} = \frac{\text{treated value - control value}}{\text{control value}} \times 100$$

Actual differences in percent were used to determine the changes in the percentage of positive cells.

These differences were plotted against time in culture, and the points joined so that any patient-to-patient variation could be seen. From the graphs it was apparent that most of the consistent changes in antigen expression had occurred by 2 days, so this time point was used to determine the statistical significance of the differences observed using the Wilcoxon matched pairs ranked sign test. A $p<0.05$ indicated that a significant change had occurred.

RESULTS

Cell viability and size after culture with αIFN

HCs, CLL and normal B lymphocytes cultured for up to 8 days in the presence or absence of either αIFN, γIFN or PMA remained more than 90% viable, as indicated by trypan blue dye exclusion. No significant differences were observed in cell size between the treated and control cells (Table 3). The cell sizes were not significantly different from those of cells directly *ex vivo* (data not shown).

HC no.	αIFN	2 days		4 days	
		Volume (fl)	Viability (%)	Volume (fl)	Viability (%)
1	-	480	90	470	90
	+	440	95	450	93
2	-	480	91	460	91
	+	440	90	430	90
3	-	440	95	440	90
	+	440	95	470	89

Table 3: Cell size and viability of HCs cultured with and without αIFN.

HC antigens unaffected by exposure to αIFN

MAbs against the following antigens were unreactive with HCs before and after 8 days culture with αIFN; CD3, CD4, CD5, CD10, CD11b, CD14, CD16, CD21, CD23, CD38 and CD71 (data not shown).

The antigens detected by the following MAbs showed no change in either the modal fluorescence per cell or the percentage of positive cells, as compared with control HCs incubated for up to 8 days without αIFN; CD11c, CD20, CD24, CD37, CD39 and CD40 (Table 4).

HC antigens reduced by exposure to αIFN *in vitro*

After 2 days of culture with αIFN the modal fluorescence per cell of CD25, light chain (LC) surface immunoglobulin (sIg) and FMC7 were all significantly reduced ($p<0.05$) (Table 5, Fig. 1). The percentage of cells positive for FMC7 was also significantly reduced ($p=0.013$) after 2 days exposure to αIFN (Table 5). In general, the reduced antigen expression persisted throughout the 8 days of culture (Fig. 1).

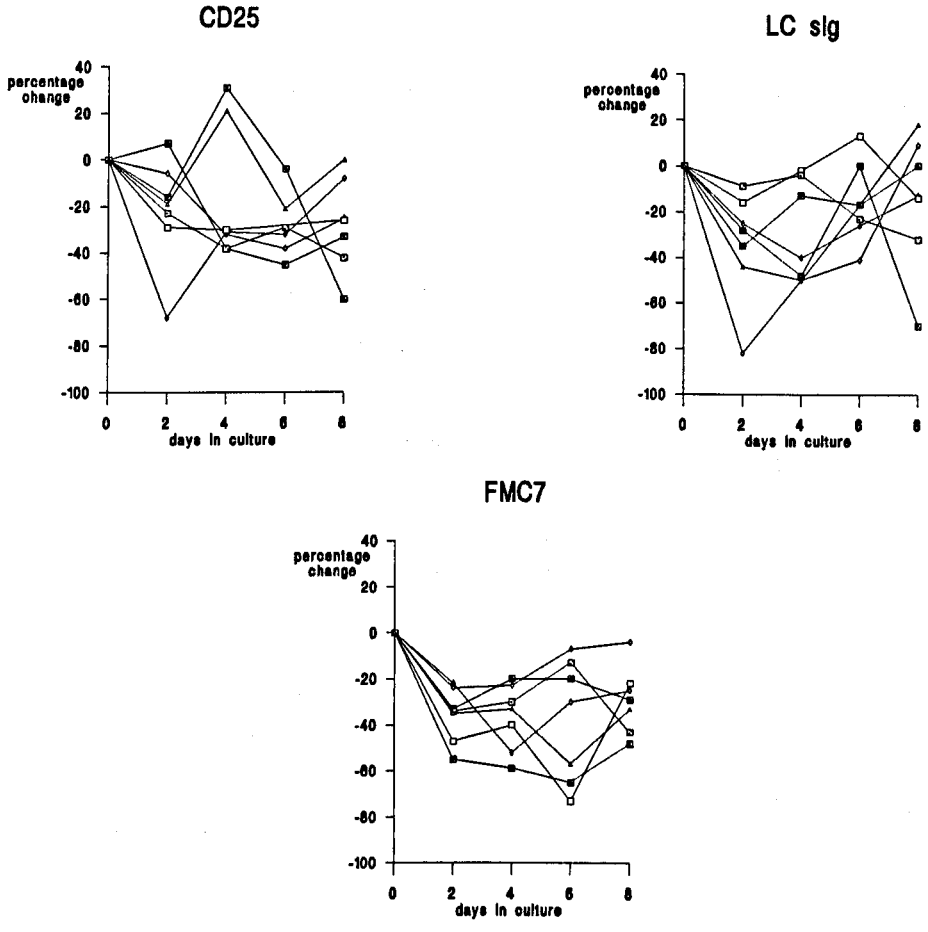


Fig. 1: Antigens decreased by exposure to αIFN.

MAb	αIFN	% ± SEM[a]	p	Mode x 10^5 ± SEM[a]	p
CD11c	-	84±3	0.38	1.3±0.1	0.69
	+	83±5		1.5±0.1	
CD20	-	96±1	0.17	3.3±0.3	0.69
	+	95±1		2.7±0.3	
CD24	-	81±3	1.0	0.9±0.1	0.94
	+	76±7		1.7±0.9	
CD37	-	94±1	0.12	2.5±0.1	0.33
	+	91±2		1.9±0.2	
CD39	-	77±4	0.11	0.8±0.1	0.33
	+	68±6		0.6±0.1	
CD40	-	81±4	0.12	1.0±0.9	0.44
	+	87±3		1.2±0.2	

Table 4: Antigens on HCs that remained unaffected by up to 8 days treatment with α IFN. a) Comparable results were obtained at 4, 6 and 8 days

The modal fluorescence per cell of CD9, CD19 and HC2, and the percentage of cells positive for CD25 and HC2, also decreased after 2 days culture with αIFN. These changes, however, were not found to be significant when taking the patient group as a whole (Table 6).

No changes were seen in either the fluorescence per cell or the percentage of positive cells during the first 24 hours of culture (data not shown).

MAb	αIFN	% ± SEM	p	mode x 10^5 ± SEM	p
CD25	-	80±4	0.09	0.9±0.01	0.05
	+	67±4		0.6±0.01	
LC sIg	-	68±8	0.7	1.7±0.6	0.012
	+	60±8		0.5±0.1	
FMC7	-	76±4	0.08	0.9±0.001	0.02
	+	53±8		0.5±0.05	

Table 5: Antigens significantly reduced after culture for 2 days with αIFN.

MAb	αIFN	%±SEM	p	Mode x 10^5±SEM	p
CD9	-	84±3	0.69	1.3±0.2	0.38
	+	79±5		1.0±0.2	
CD19	-	87±3	0.44	1.3±0.1	0.09
	+	86±2		1.0±0.1	
HC2	-	67±9	0.14	0.6±0.05	0.4
	+	48±10		0.5±0.05	

Table 6: Antigens non-significantly reduced after 2 days exposure to αIFN.

HC antigens increased by exposure to αIFN *in vitro*

The intensity of staining with the anti-MHC (class I & II) MAbs was increased after 12 hours of culture with αIFN, and was maximal between 24 and 48 hours (Fig. 2). This change was significant ($p<0.05$) at 2 days, except in the case of class I MHC

(Table 7). However, since over 90% of HCs were positive for monomorphic MHC (class I and II) and DR before exposure to αIFN the increase in modal fluorescence was not accompanied by an increase in the number of positive cells. Due to the wide variation in the number of cells positive for DP (7-90%) and DQ (14-89%) the changes in the percentage of cells positive for these antigens were not significant when taking the patient group as a whole.

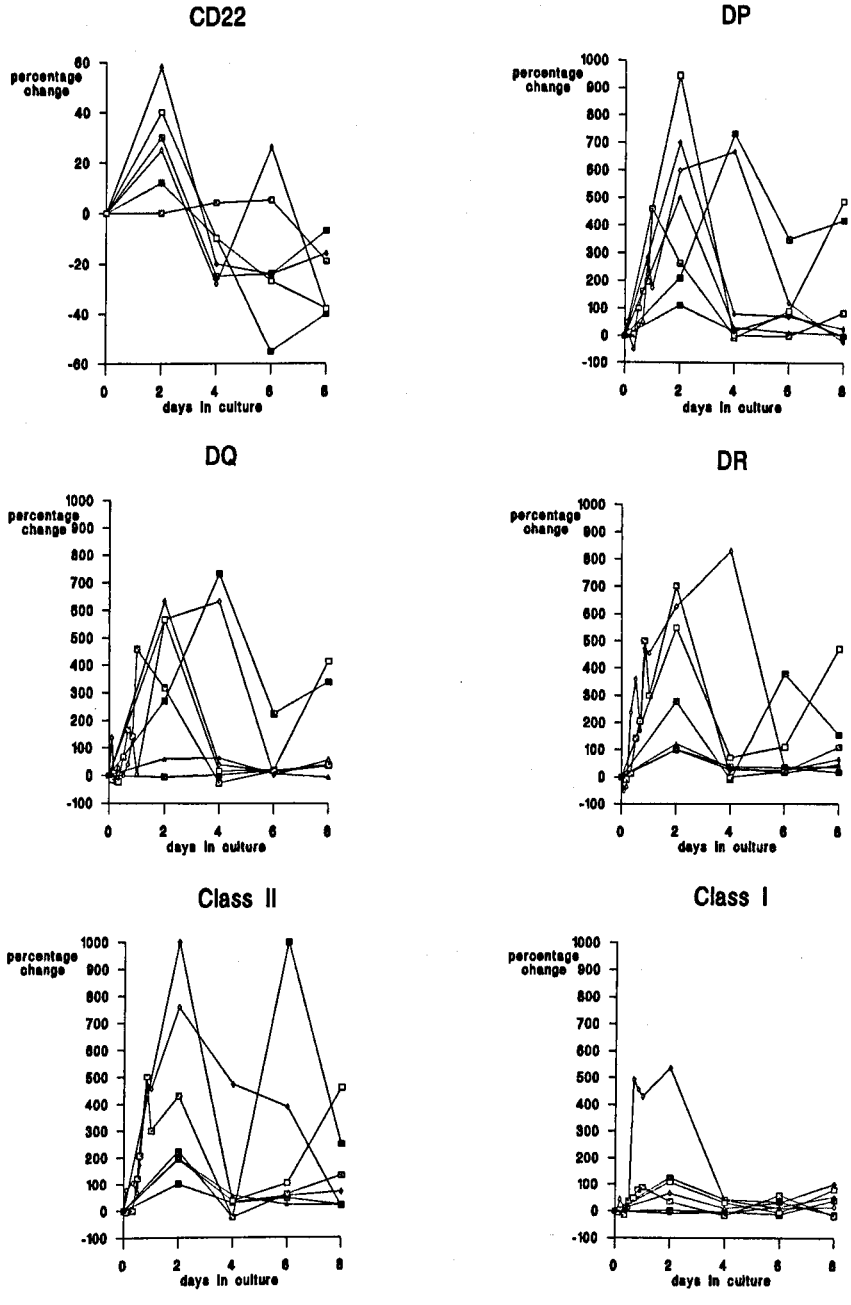


Fig. 2: Increased antigen expression in HCs in response to αIFN.

MAb	αIFN	%±SEM	p	Mode x 10^5±SEM	p
DP	-	56±11	0.2	0.5±0.07	0.002
	+	73±6		2.7±0.5	
DQ	-	49±10	0.34	0.6±0.01	0.01
	+	67±6		2.2±0.3	
DR	-	84±2	0.44	1.1±0.2	0.002
	+	86±3		5.4±0.8	
Class II	-	90±2	0.09	1.4±0.2	0.002
	+	85±3		6.8±1.1	
Class I	-	98±0	0.9	7.9±1.9	0.13
	+	96±1		13±2	
CD22	-	89±3	0.93	1.0±0.1	0.03
	+	89±3		1.4±0.1	

Table 7: Antigens increased by exposure to αIFN.

αIFN also caused increased expression of CD22 after 2 days exposure to αIFN with respect to modal fluorescence, but not percentage positivity (Table 7). This antigen was not tested for changes in expression during the first 24 hours of culture.

Antigen expression on HCs from a patient who is clinically resistant to αIFN

None of the above changes in antigen expression were observed in the HCs of this patient (Table 8). No other changes in antigen expression were seen (data not shown). However, the phenotype of HCs from this patient closely resembled the αIFN treated cells from other patients in that HLA expression was markedly higher and CD25, FMC7 and light chain sIg were lower than in other cases.

HC antigen expression after exposure to γIFN *in vitro*

Light chain sIg was significantly reduced ($p<0.05$) after 2 days of culture with γIFN. This reduction persisted throughout the period of culture (Fig. 3; Table 9).

MAb	αIFN	%	Mode x 10^5
CD19	-	96	1.3
	+	94	1.1
CD22	-	93	1.3
	+	90	0.8
CD25	-	33	0.4
	+	50	0.4
DP	-	98	2.9
	+	98	2.7
DQ	-	99	4.0
	+	98	3.2
DR	-	95	5.6
	+	96	4.3
Class II	-	96	7.0
	+	96	5.4
Class I	-	98	5.4
	+	99	5.4
FMC7	-	57	0.5
	+	60	0.4
HC2	-	12	<0.3[a]
	+	7	<0.3[a]
LC sIg	-	88	0.8
	+	88	0.8

Table 8: Effect of αIFN on HCs from the patient who is resistant to αIFN *in vivo*.

a) 0.3 x 10^5 is the limit of detection by FACS.

With the exception of light chain sIg, the changes produced by γIFN were significantly different ($p<0.05$) from those produced by αIFN (data not shown).

HC antigen expression after exposure to PMA

Treatment with PMA also induced changes in antigen expression. In contrast to treatment with α and γIFN, where the changes were most marked at 2 days, the changes induced by PMA were greatest at 4 days. PMA induced decreased expression of FMC7, CD19, CD20 and light chain sIg (Fig. 4). Increased expression, as with

αIFN, was seen in the HLA antigens, with DP and DQ being increased both in terms of modal fluorescence and the number of positive cells; while DR and monomorphic MHC class I and II were only increased in terms of modal fluorescence (Fig. 5).

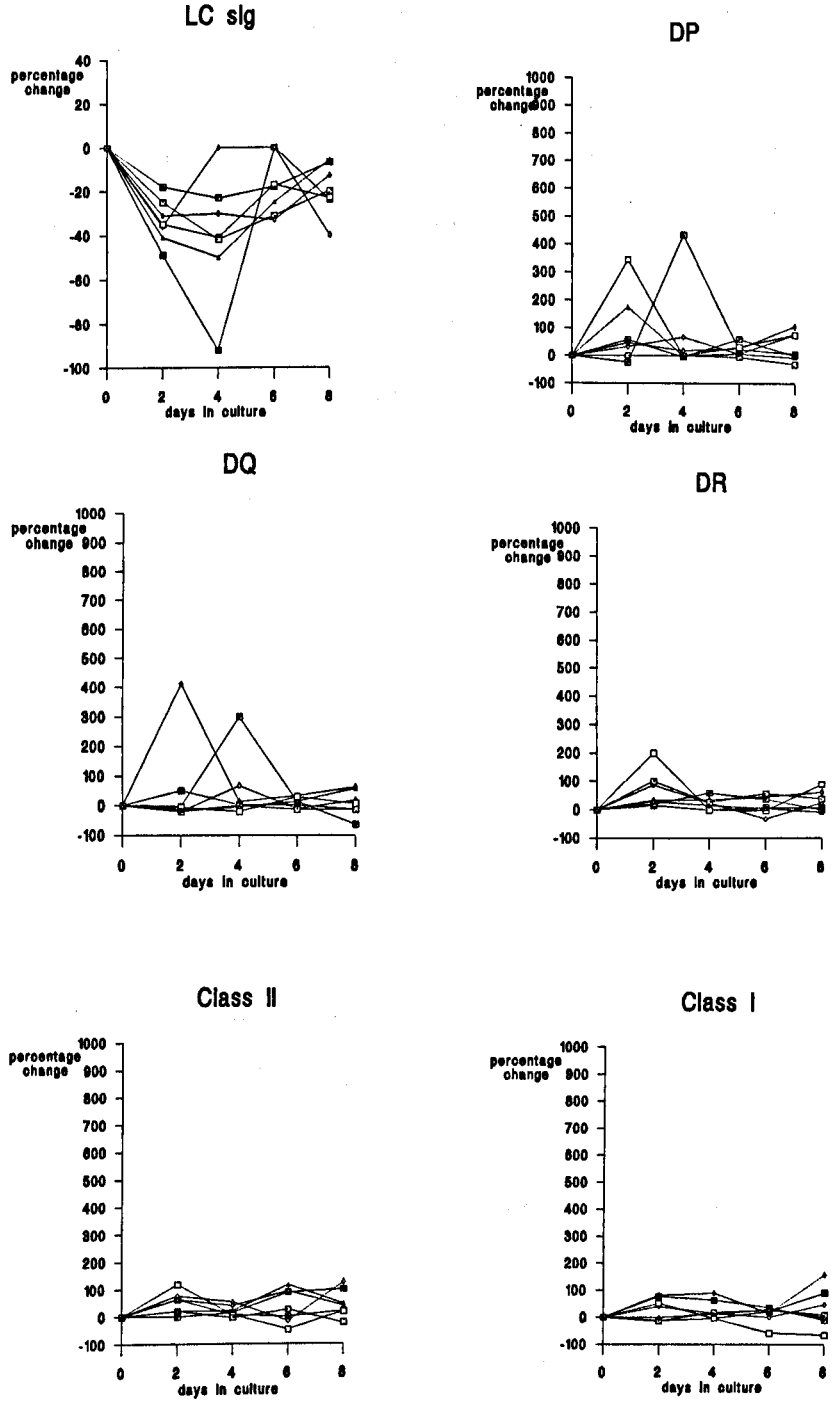


Fig. 3: HC antigens altered by exposure to γIFN.

MAb	γIFN	% ± SEM	p	Mode x 10^5 ± SEM	p
LC sIg	-	68±6	0.02	1.7±6	0.013
	+	41±6		0.5±0.07	
DP	-	56±11	0.25	0.5±0.07	0.11
	+	70±6		1.0±0.04	
DQ	-	49±10	0.31	0.6±0.02	0.48
	+	61±8		0.9±0.03	
DR	-	93±8	0.7	1.0±0.2	0.11
	+	96±4		1.8±0.3	
Class II	-	90±2	0.89	1.4±0.02	0.2
	+	94±5		2.1±0.4	
Class I	-	98±0	0.22	7.8±1.6	0.4
	+	97±1		11±1	

Table 9: Changes in antigen expression in response to γIFN.

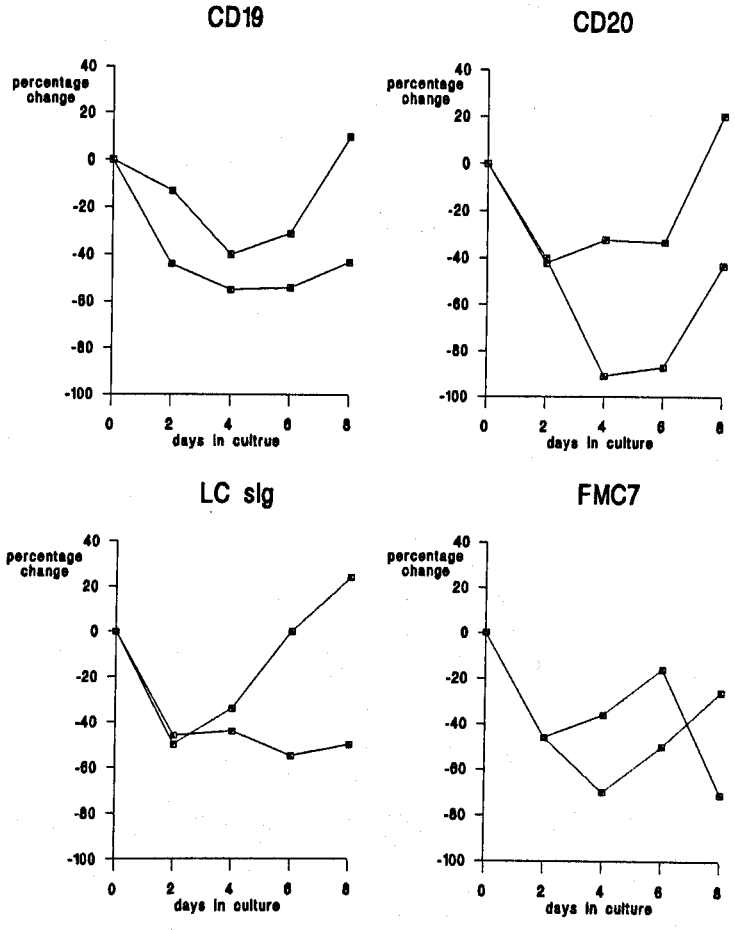


Fig. 4: HC antigens decreased in response to PMA.

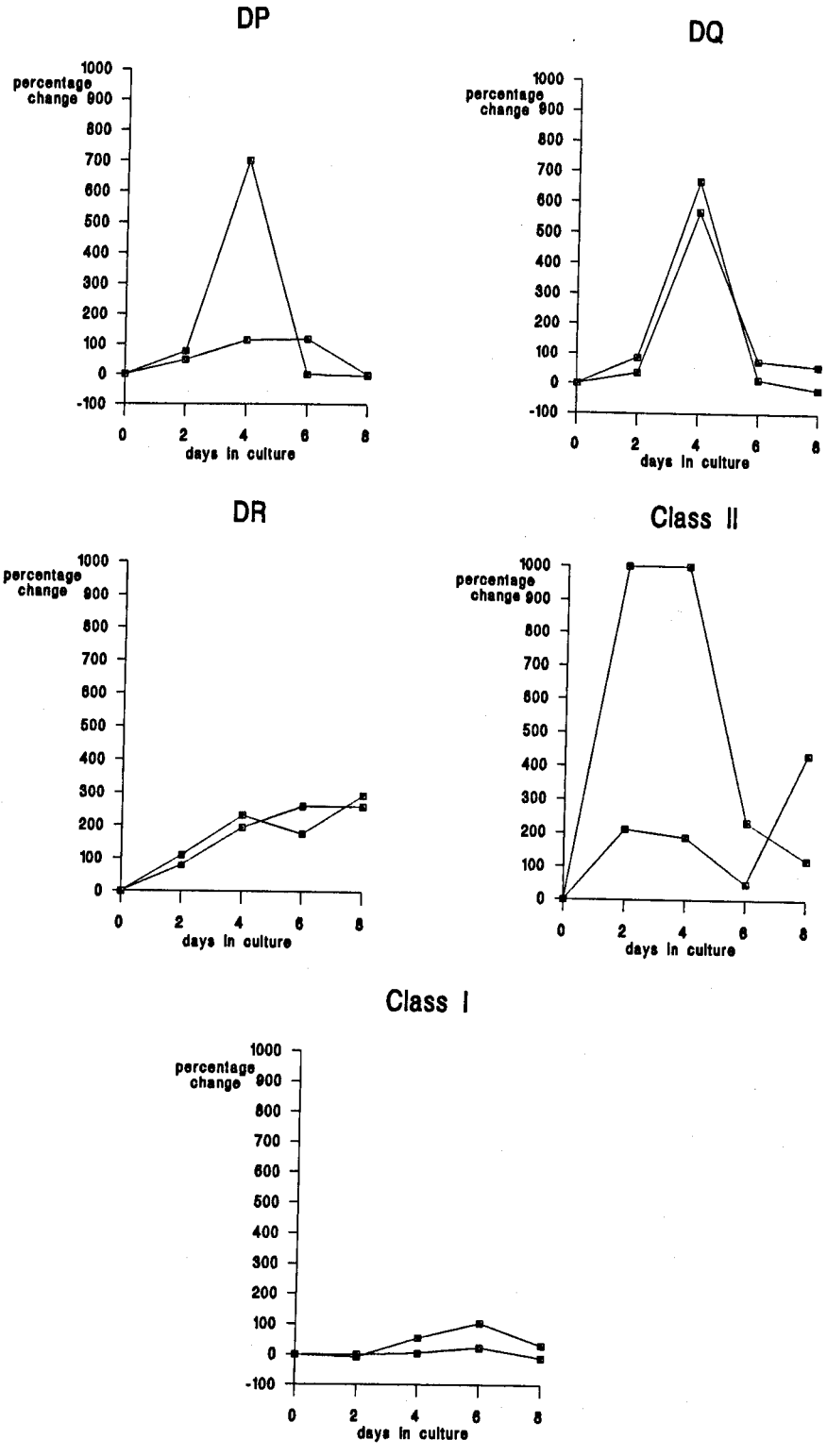


Fig. 5: HC antigens increased by exposure to PMA.

CLL antigen expression after exposure to αIFN

αIFN treatment of CLL cells did not alter the expression of any of the antigens altered on HCs ($p<0.05$; Table 10). No consistent changes were observed in any of the other antigens tested (data not shown).

MAb	αIFN	% ± SEM[b]	p	Mode x 10^5 ± SEM[b]	p
CD9	-	36±15	0.56	0.4±0.01	0.5
	+	55±38		0.5±0.02	
CD19	-	80±6	0.6	0.4±0.9	0.39
	+	89±3		0.5±0.02	
CD22	-	29±12	0.9	0.3±0.01	0.39
	+	30±20		0.3±0.04	
CD25, FMC7, HC2, LC sIg[a]	-	0		0	
	+	0		0	
DP	-	60±17	0.2	0.8±0.2	0.76
	+	87±5		0.9±0.2	
DQ	-	52±17	0.5	0.5±0.1	0.25
	+	68±17		0.9±0.3	
DR	-	88±4	0.2	1.4±0.3	0.11
	+	92±2		2.0±0.4	
Class II	-	94±2	0.3	1.8±0.4	0.06
	+	92±1		2.6±0.6	
Class I	-	97±1	0.5	3.2±0.5	0.78
	+	98±1		3.5±0.5	

Table 10: Antigen expression on CLL at 2 days cells in response to αIFN. a) Antigens also negative at time 0. b) Antigen expression remained similar at other time points tested.

Antigen expression on normal B lymphocytes after exposure to αIFN

αIFN caused increases in both the modal fluorescence and expression of class I MHC on normal B cells after 2 days of culture (Table 11). No changes were seen in the expression of other antigens altered by αIFN in HCs (Table 11).

MAb	αIFN	% ± SEM	Mode x 10^5 ± SEM
CD19	-	90±3	0.5±0.1
	+	91±4	0.5±0.1
CD22[a]	-	95	0.6
	+	98	0.6
DP[a]	-	98	0.8
	+	99	0.9
DQ[a]	-	91	0.5
	+	99	0.7
DR	-	83±13	1.5±0.1
	+	80±19	2.9±0.6
Class II[b]	-	97	1.8
	+	99	2.4
Class I	-	100±1	1.6±0.6
	+	100±1	2.3±1.2

Table 11: Effect of αIFN on antigen expression by normal B cells. a) Only one tonsil was positive for these antigens. b) Only one tonsil was tested for this antigen.

DISCUSSION

The results presented in this chapter clearly demonstrate that, after *in vitro* exposure to αIFN, HCs show greatly enhanced expression of MHC class I and II (DP, DQ and DR), and to a lesser extent CD22. This increase in HLA DR confirms the results of other studies (Baldini *et al* 1986; Gamliel *et al* 1988). αIFN does not, in general, increase class II expression (Burrone & Milstein 1982; Collins *et al* 1984). This was confirmed in the present study, as αIFN did not cause increases in MHC class II on either normal or CLL B lymphocytes.

αIFN also caused a significant reduction in the expression of CD25, light chain sIg and FMC7 on HCs; although CD9, CD19 and HC2 expression were also reduced, this reduction was not statistically significant. In contrast, no changes in antigen expression were seen in the HCs of the patient whose cells proliferated in the absence

of added growth factors *in vitro.* In fact, the phenotype of the HCs from this patient closely resembled those of the other cases following αIFN treatment. This may indicate that the HCs of this patient had differentiated past the stage at which αIFN exerts its effect. The fact that the effects of αIFN are dependent on the stage of B cell differentiation was confirmed by the fact that, apart from the increased class I MHC expression, the cytokine had no effect on the antigen expression of either CLL or normal B lymphocytes.

The changes in antigen expression reported here contrast with those of the previous study (Baldini *et al* 1986), which did not report any changes in the expression of CD19 or CD25 after culture with αIFN (they did not examine any of the other antigens reported, in this chapter, to be altered by culture with αIFN). The reasons for this apparent discrepancy are not clear, but may be at least partly attributable to the fact that their culture conditions employed FCS which was found in the present study to partially inhibit the increase in HLA DR.

γIFN treatment of HCs also caused changes in antigen expression. Although MHC class II expression was enhanced, this increase was not statistically significant, and was much less than that induced by αIFN. A decrease in the expression of light chain sIg comparable to that induced by αIFN was also observed. The significant changes specific to αIFN were therefore reduced levels of CD25 and FMC7 together with increased CD22 and HLA expression. Since γIFN is ineffective in the treatment of HCL (Niederle *et al* 1986), it is the specific changes produced by αIFN that are likely to be important in relation to the possible mechanism(s) of action of αIFN in the disease.

Increased expression of both class II MHC and CD22 is a feature of B cell activation (Engel *et al* 1987; Godal *et al* 1985; Pezutto *et al* 1987a). Increased expression of class II is also seen on HCs following activation (Gamliel *et al* 1990; Griffiths & Cawley 1991). However, the changes in antigen expression on HCs cannot be simply attributed to activation since other well known B cell activation antigens, for example CD23 and CD71 (Guy & Gordon 1987), were not increased by exposure

to αIFN. Still other B cell activation antigens, for example CD25 and HC2 (Bentaboulet *et al* 1987; Posnett *et al* 1982; 1984), were reduced by incubation with αIFN. As CD19 acts as a receptor for a growth inhibitory factor (Pezutto *et al* 1987b), and CD22 as a co-stimulatory factor in B cell activation (Pezutto *et al* 1987a), as well as being involved in adhesion (Wilson *et al* 1991), these changes in HC antigen expression may alter the ability of HCs to respond to external stimuli.

The reduction of CD19, CD25 and FMC7 could all be explained in terms of differentiation, since these antigens are all lost in the latter stages of B lymphocyte differentiation (Zola 1987). However, such differentiation must be incomplete because the HCs do not acquire the CD38 plasma cell antigen, and since fully mature plasma cells completely lack CD19, CD22 and class II. As HCs show no propensity to become plasma cells (Anderson *et al* 1985; Griffiths & Cawley 1990), it is not surprising that αIFN treated HCs do not acquire characteristic plasma cell antigens. αIFN also causes the differentiation of other cell types, including monocytes (Perussia *et al* 1987). Although these changes in antigen expression are difficult to interpret, they may indicate that αIFN induces partial activation/differentiation of HCs. The fact that PMA, which induces B cell activation/differentiation (Engel *et al* 1987), caused antigenic changes similar to those produced by αIFN lends support to this hypothesis.

In conclusion, αIFN causes a marked increase in the expression of MHC class I and II antigens, together with reduced expression of CD25, light chain sIg and FMC7. These changes are specific to HCs and, with the exception of light chain sIg, to αIFN. The results discussed in this Chapter therefore show that, *in vitro*, αIFN causes partial activation/differentiation of HCs, thus changing the ability of HCs to respond to external stimuli; which could result in a reduced life span for HCs *in vivo*. This may, at least in part, explain the beneficial effects of αIFN treatment *in vivo*.

THE EFFECT OF αIFN ON *C-MYC* AND *C-FOS* EXPRESSION BY HCS

CHAPTER 2

INTRODUCTION

HCs express a number of proto-oncogenes; although at the time of this study (1987) only two of these *c-myc,* and *c-fos,* were known to be regulated by the cytokine αIFN (Jonak & Knight 1986), and to be involved in the signalling pathways which regulate activation/differentiation of cells. Since *c-fos* is found in higher amounts in HCL than in other B-cell leukaemias (Pinto *et al* 1987), regulation of this proto-oncogene is likely to have more profound effects on the pathogenesis of HCL. The expression of high levels of *c-fos* by HCs reflects the fact that they are highly differentiated, as increased expression of this oncogene parallels cell maturation (Mavilo *et al* 1987). *C-fos* is a component of the signalling pathways for activation (Müller *et al* 1984), differentiation (Ruther *et al* 1985; Erandi *et al* 1986), and the regulation of MHC antigen expression (Kushtai *et al* 1988; Barizilay *et al* 1987). αIFN can both decrease (Friedman 1986) and induce *c-fos* expression (Kushtai *et al* 1988). Decreased *c-fos* expression is usually followed by growth arrest (Jonak & Knight 1986).

HCs express low levels of *c-myc* (Lehn *et al* 1986), this reflects the fact that they are not actively dividing cells (Jonak & Knight 1986). *C-myc* is also a component of the signalling pathway for cell growth (McNerney *et al* 1985), and is decreased in response to αIFN (Resnitsky *et al* 1986). As with *c-fos* this decrease is followed by growth arrest (Einat *et al* 1985).

The effects of αIFN on *c-myc* and *c-fos* protein expression in HCs was therefore investigated. It was hoped that this would shed some light on whether the antigenic changes induced by αIFN on HCs, reported in Chapter 1, were due to activation or differentiation of HCs, and to give further insight into the mechanisms by which αIFN acts in HCL. The effect of αIFN on *c-myc* and *c-fos* protein expression on growth factor stimulated HCs was also examined to establish what the effects of αIFN on the clonaly expanding HC population *in vivo* might be. In view of the relationship between decreased *c-myc* and *c-fos* expression and growth arrest the

proportion of cells in each phase of the cell cycle was measured in parallel with the examination of proto-oncogene expression.

PATIENTS AND METHODS

Patients

PB HCs from 12 patients with HCL were studied. All the patients studied had typical disease; although one was unusual in that HCs from this patient were refractory to the effects of αIFN treatment *in vivo* and underwent proliferation in the absence of added growth factors *in vitro*. The PB of 4 patients with CLL was also studied, together with lymphocytes from 2 normal tonsils.

Cell preparation

Peripheral blood mononuclear cells (PBM) from HCL and CLL patients were isolated by density gradient centrifugation over lymphoprep. The PBM obtained were then frozen in liquid nitrogen (as in Chapter 1).

In general, frozen cells were employed in the studies described, but for one patient both fresh and frozen HCs were examined and comparable results obtained (data not shown).

T-cells and monocytes were removed by AET-SRBC rosetting and plastic adherence respectively where they constituted >5% of the PBM (as in Chapter 1). This resulted in >95% CD19 positive B lymphocytes (CD3<5%, CD14<5%).

Lymphocytes were removed from tonsils by pushing the tissue through a fine wire gauze followed by density gradient centrifugation over lymphoprep (as for splenic HCs in Chapter 1). T lymphocytes and monocytes were removed as above. The resulting B lymphocyte population was >98% CD19 positive (CD3<1%, CD14<1%). Tonsil B lymphocytes were separated by density gradient centrifugation over 62.5% percoll into their activated (low density) and resting (high density) components.

Cell lines

Daudi cells were used as a control system since the decrease in *c-myc* expression in response to αIFN in these cells is well documented. HL60 cells express both the onco-proteins *c-myc* and *c-fos*, they were therefore used as a positive control of the staining procedure.

These cell lines were kept in continuous culture in RPMI 1640 medium containing 10% FCS, 2mM L-glutamine 100U/ml penicillin, and 100μg/ml streptomycin (Complete-RPMI, Flow Labs.).

Culture of HCs

All cultures were performed at a cell concentration of 2 x 10^6/ml in 24 well tissue culture plates (Falcon), in complete-RPMI at 37°C in 5% CO_2 in air.

Establishing time course of experiments. Previously reported changes in the mRNA coding for the proto-oncogenes *c-myc* and *c-fos* occurred within the first 24 hours of culture (Conscience *et al* 1986), and in some cases these effects were transient (McNerney *et al* 1985). HCs from two patients were therefore cultured in the presence and absence of 100U/ml αIFN (Wellcome) for 24 hours and sampled at 2 and 4 hours, then every 4 hours up to 24 hours. However, no changes in the expression of either the *c-myc* or *c-fos* proteins was seen.

As a positive control Daudi cells were cultured with and without 100U/ml αIFN and sampled every 2 hours for 8 hours and then every 24 hours up to 4 days. Under the above experimental conditions changes in *c-myc* and *c-fos* protein expression were seen at 2 and 3 days (Fig. 1).

Titration of αIFN. αIFN was titrated between 100 and 1000U/ml on the HCs which were sampled at 1, 2, 4 and then every 24 hours up to 4 days. No difference in the expression of either the *c-myc* or *c-fos* proteins was seen with any of these doses (Fig. 2). Since 100U/ml was sufficient to induce the changes in oncoprotein expression on Daudi cells this dose was chosen in order to maintain consistency of doses with those used in Chapter 1, and because it is within the therapeutic range.

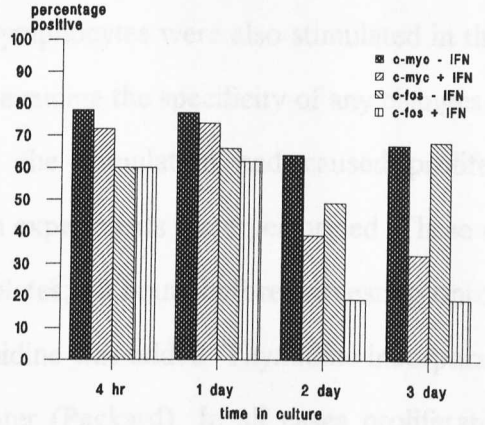


Fig. 1: Effect of αIFN on *c-myc* and *c-fos* protein expression by Daudi cells.

HCs from the 5 patients were therefore cultured either with or without the addition of 100U/ml αIFN for between 4 and 6 days; the cells were sampled every 24 hours for 6 days in 4 patients and for 4 days in one patient.

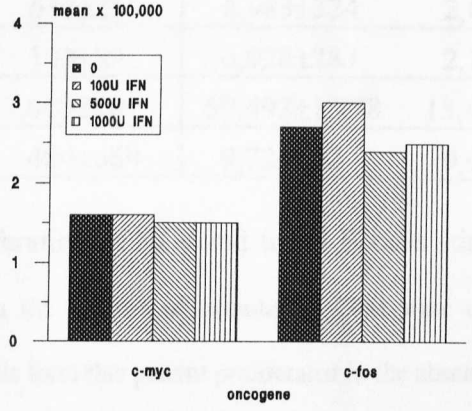


Fig. 2: Effect of different doses of αIFN on *c-myc* and *c-fos* protein expression by HCs.

Stimulation of HCs. In addition HCs were also examined in five cases where they had been stimulated to proliferate with 10% B cell growth factor (BCGF) and 10μg/ml of sepharose conjugated anti-μ. These concentrations of growth factors had been found to give an optimal proliferative signal to HCs (Griffiths & Cawley 1990). HCs stimulated in this way were cultured both with and without 100U/ml α or γIFN and harvested for oncoprotein staining at 4 and 24 hours, and then at 4, 5 and 6 days.

Normal tonsil B lymphocytes were also stimulated in this way and treated with α and γIFN in order to examine the specificity of any changes seen in HCs.

To confirm that the stimulation had caused proliferation parallel tritiated thymidine incorporation experiments were performed. These cultures were performed in triplicate in 96 well plates; 18 hours before harvesting onto glass fibre filter papers 0.5μCi of tritiated thymidine was added. Thymidine incorporation was measured on a liquid scintillation counter (Packard). In all cases proliferation, as indicated by the uptake of tritiated thymidine, was seen (Table 1).

Patient no.	3 day[a]	9 day[a]		
		No IFN	αIFN	γIFN
1	265±52[b]	5,759±340	1,900±170	6,744±592
2	3,027±336[b]	16,992±4,541	8,444±1,901	11,005±941
3	163±15[b]	7,652±294	1,216±106	6,978±492
4	638±25[b]	6,983±324	2,061±118	6,106±419
5	103±8[b]	6,928±283	2,106±203	5,218±483
7[c]	3,809±20	59,492±1,778	13,409±342	56,640±369
Tonsil	484±66[b]	9,721±22	9,958±718	11,324±913

Table 1: Proliferation of HCs and tonsil B cells stimulated with BCGF and anti-μ.a) CPM±SEM. b) In the absence of stimulation CPM were of similar values at 6 and 9 days. c) Unstimulated (Cells form this patient proliferated in the absence of exogenous stimuli)

Preparation of nuclei

Nuclei were extracted according to the method of Baines *et al* (1987): Briefly $1x10^6$ cells were fixed between 1 and 6 days in absolute methanol to permeabilise the cells. The different time of fixation does not affect staining or oncogene expression (Baines, personal communication). The cells were washed and incubated at room temperature with a trypsin solution to digest the cell membrane and cytoplasm. The digestion was then stopped using trypsin inhibitor and the RNA was digested using RNAase; since propidium iodide (PI) binds to both DNA and RNA, any RNA present would affect the cell cycle data. The reaction was stopped by adding an ice cold

solution containing an excess of the nuclei stabilising molecule spermidine tetrahydrochloride (STC) and the nuclei were pelleted (Appendix 2.1).

Staining of nuclei

Antibodies. A mouse MAb to the *c-myc* protein (DCM905, Cambridge research Biochemicals (CRB)) and a sheep polyclonal antibody to the *c-fos* protein (DCP850, CRB) were used.

Antibody concentrations. The antibodies were titrated between 5 and 50μg/ml on HL60 cells and saturating concentrations were used for subsequent experiments. The antibody concentrations used were 10μg/ml for *c-myc* and 5μg/ml for *c-fos*.

Specificity of the antibodies. The specificity of the two antibodies in this system was shown using displacement experiments; the antibodies were pre-incubated for one hour prior to staining either with their immunising peptide, or with an inappropriate peptide (*c-fos* peptide (OP-11-3020 CRB) for the *c-myc* MAb and *c-myc* peptide (OP-11-3030 CRB) for the *c-fos* antibody). These antibodies were then used for staining as before; the modal fluorescence per cell was reduced by 30-50% with the appropriate peptide relative to the non-treated controls, and no displacement was seen with the inappropriate peptides (Fig. 3).

Staining. Nuclei were incubated for 1 hour on ice with the antibodies to either the *c-myc* or *c-fos* proteins, with mouse IgG1 or sheep serum as first layer controls. After washing with STC, the nuclei were stained with the appropriate FITC conjugated second layer, either goat anti-mouse or rabbit anti-sheep, at 25μg/ml, for 30 minutes on ice. The nuclei were then washed and stained with PI for a minimum of 5 minutes.

FACS analysis

The nuclei were analysed on a FACS analyser I (Becton Dickinson) using linear amplification for both green (530nm) and red (595nm) fluorescence. Data were recorded on a Consort 30 computer (BD). The percentage of positive cells and the

mean green fluorescence were recorded for each antibody. The mean fluorescence was converted into molecules of FITC bound per cell by reference to a standard line drawn using beads of known fluorescence and volume (Flow cytometry standards corporation).

The percentage of cells in each phase of the cell cycle was calculated using BD software on the consort 30 computer.

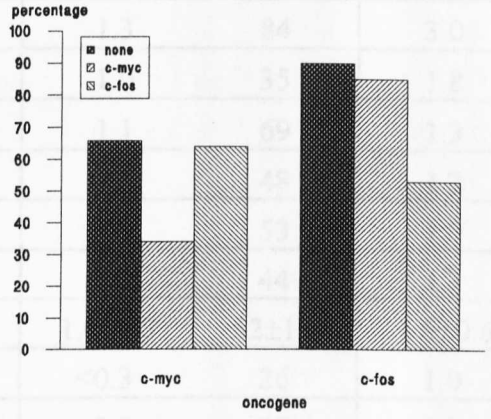


Fig. 3: Displacement of *c-myc* and *c-fos* antibody binding on HL60 cells by their immunising peptides.

Reproducibility of the technique

As a positive control HL60 nuclei were stained along with every batch of HCs analysed; this also enabled the reproducibility of the technique to be monitored. Variations in the parameters measured were calculated by dividing the standard error by the mean for all the positive controls; the values for molecules per cell varied by 16%, for percentage of positive cells by 11%, and for cell cycle phase by 3%.

RESULTS

C-myc and *c-fos* protein levels in HCL and CLL cells tested directly *ex vivo*

The levels of *c-myc* and *c-fos* protein in HCs varied greatly both with respect to the percentage of positive cells and to the number of molecules per cell. In general, however, the intensity of expression of *c-myc* was lower then that of *c-fos* (Table 2).

Cell no.	*c-myc*		*c-fos*	
	mean x 10^5	%	mean x 10^5	%
HC 1	0.7	45	1.7	60
HC 2	1.5	57	1.6	83
HC 3	0.8	53	1.2	65
HC 4	1.6	63	2.6	88
HC 5	<0.3[a]	14	1.1	56
HC 6	1.2	55	1.7	67
HC 7	1.3	84	3.0	87
HC 15	1.0	35	1.8	44
HC 16	1.1	69	1.3	73
HC 17	1.0	48	1.2	65
HC 18	1.0	53	1.0	33
HC 19	1.0	44	1.3	78
Mean HC	1.0±0.3	52±17	1.6±0.6	66±16
CLL 1	<0.3	26	1.0	54
CLL 2	<0.3	47	0.8	69
CLL 3	0.9	64	1.4	65
CLL 4	1.0	76	3.4	85
Mean CLL	0.7±0.2	53±9	1.7±1.0	56±5
Tonsil 1[b]	<0.3	5	1.2	35
Tonsil 2[b]	0.9	48	1.4	49
Tonsil 3[c]	1.0	63	1.6	61
Tonsil 4[c]	0.7	62	1.7	58

Table 2: Oncoprotein expression in B cells from HCL CLL and tonsil measured directly *ex vivo*. a) 0.3 x10^5 is the limit of detection by FACS. b) High density fraction. c) Low density fraction.

In the patient whose HCs showed proliferation in the absence of added growth factors *in vitro* (HC 7, Table 2), *c-myc* levels were higher than in the other patients in terms of percentage of positive cells, but not of mean fluorescence per cell (Table 2). Levels of *c-fos* in this patient's HCs were higher than in the other patients, (Table 2).

Levels of *c-myc* and *c-fos* also varied widely in CLL cells. The levels of *c-myc* were comparable in percentage terms with those seen in HCs, but displayed lower

mean fluorescence per cell. The levels of *c-fos* were similar to those seen in HCs (Table 2).

The percentage of cells positive for both *c-myc* and *c-fos* were, as expected, lower in the resting than in the activated tonsil B lymphocyte fraction (Table 2).

a. *c-myc*

Patient	αIFN	1 day		4 day[a]	
		Mean x 10^5	%	Mean x 10^5	%
1	-	1.4	62	1.3	40
	+	1.2	55	1.3	46
4	-	1.6	83	1.7	88
	+	1.6	92	1.5	90
5	-	<0.3	24	<0.3	27
	+	<0.3	23	<0.3	28
6	-	1.5	57	1.4	46
	+	1.5	51	1.4	47

b. *c-fos*

HC no.	αIFN	1 day		4 day[a]	
		Mean x 10^5	%	Mean x 10^5	%
1	-	1.7	63	1.3	68
	+	1.6	67	1.3	63
4	-	2.6	88	3.3	86
	+	2.5	91	2.7	80
5	-	1.3	82	1.0	75
	+	1.5	76	1.1	87
6	-	1.6	78	1.7	59
	+	1.7	82	1.5	65

Table 3: Effects of αIFN on *c-myc* and *c-fos* expression in unstimulated HCs.

a) Levels were similar at other time points tested.

Effects of αIFN on *c-myc* and *c-fos* protein expression in HCs

In the absence of αIFN, levels of the *c-myc* and *c-fos* proteins remained constant throughout the 6 days of culture. Exposure to αIFN produced no consistent changes in these levels either during repeated testing during the first 24 hours or on daily testing for up to 6 days (Table 3).

Effects of αIFN on *c-myc* and *c-fos* protein expression in the HCs of patient 7. In common with the other patients' cells, HCs from this patient showed no consistent changes in the levels of either the *c-myc* or *c-fos* proteins in the absence of αIFN (Table 4). However, at 5 days αIFN caused a transient reduction in both the levels and the percentage of cells expressing the *c-myc* and *c-fos* proteins (2/2 experiments; Table 4); whereas γIFN treatment had no effect on oncoprotein expression in this patient's HCs throughout the period of culture (Table 4).

Onco-gene	IFN	0		4 day		5 day		6 day	
		Mean x 10^5	%	Mean x 10^5	%	Mean x 10^5	%	Mean x 10^5	%
c-myc	-	1.0	48	1.6	80	1.8	83	2.0	53
	α			1.4	62	<0.3	4	1.8	86
	γ			1.6	57	1.9	50	1.5	61
c-fos	-	1.3	65	3.6	93	4.3	90	4.4	81
	α			3.6	86	3.9	62	4.0	89
	γ			3.6	98	3.8	85	4.0	86

Table 4: Effect of IFN on HCs from patient 7. (One representative case)

Effect of α and γIFN on *c-myc* and *c-fos* protein levels in stimulated HCs

In the five patients studied (Nos. 2-6) stimulation with BCGF and anti-μ caused HCs from all patients to increase the amounts of both the *c-myc* and *c-fos* proteins. However, the time course of this increase differed between patients: HCs from patients 2-5 increased their *c-myc* and *c-fos* protein levels within 4 hours of

stimulation, and this increase in expression persisted throughout the period of culture (Table 5 & 6). In patient 6, the increase occurred 5 days after stimulation (Table 7).

a. *c-myc*

HC no.	IFN	0		4hrs		1 day		3 day[a]	
		Mean x 10^5	%	Mean x 10^5	%	Mean x 10^5	%	Mean x 10^5	%
2	-	1.5	57	2.1	74	2.0	67	2.1	76
	α			0.7	65	0.8	55	2.1	75
	γ			2.0	75	2.0	73	2.2	79
3	-	0.8	53	1.6	74	1.6	78	1.7	79
	α			0.7	25	0.6	20	1.8	63
	γ			1.7	77	1.7	79	1.8	75
4	-	1.6	63	2.5	84	2.5	83	2.8	87
	α			1.6	31	2.3	62	2.6	87
	γ			2.4	56	1.5	23	2.6	87

b. *c-fos*

HC no.	IFN	0		4hrs		1 day		3 day[a]	
		Mean x 10^5	%	Mean x 10^5	%	Mean x 10^5	%	Mean x 10^5	%
2	-	1.6	83	2.8	86	2.9	83	3.2	89
	α			1.5	67	2.3	67	3.3	89
	γ			2.9	87	2.9	85	2.8	86
3	-	1.2	65	2.5	86	2.6	83	2.7	86
	α			1.7	59	1.2	37	2.2	69
	γ			2.5	83	2.5	84	2.5	88
4	-	2.0	44	3.4	96	3.5	97	3.2	92
	α			2.6	22	2.9	63	3.2	88
	γ			3.6	91	2.1	25	3.2	95

Table 5: Effect of IFN on the early increase oncoprotein expression caused by BCGF and anti-μ stimulation of HCs; delayed increase in oncoprotein expression induced by αIFN. a) Levels remained constant until 6 days.

Onco-gene	IFN	0		4 day[a]		5 day		6 day	
		Mean x 10^5	%	Mean x 10^5	%	Mean x 10^5	%	Mean x 10^5	%
c-myc	-	<0.3	14	1.5	54	1.4	67	1.3	66
	α			1.3	62	0.8	22	1.3	49
	γ			1.4	60	1.5	65	1.1	56
c-fos	-	1.1	56	2.6	71	3.5	64	2.6	54
	α			2.3	60	1.8	59	2.6	65
	γ			3.0	57	2.6	75	2.2	69

Table 6: Effect of IFN on the early increase in oncoprotein expression caused by BCGF and anti-μ in the HCs of patient 5. a) Levels remained constantly increased up to this time.

Effect of αIFN. αIFN treatment affected the levels of the *c-myc* and *c-fos* proteins in all 4 patients whose HCs showed an early increase in their expression as a result of stimulation; in three of the patients (Nos. 2-4, Table 5) the increase in expression was delayed by αIFN, whereas in the other patient (No. 5, Table 6) αIFN caused a transient decrease in expression at 5 days, similar to that seen in the HCs of patient no. 7. In the patient (No. 6), who showed a late increase in oncoprotein expression as a result of stimulation, αIFN had no effect on the increase in *c-myc* or *c-fos* expression (Table 7).

Onco-gene	IFN	0		4 day[a]		5 day		6 day	
		Mean x 10^5	%	Mean x 10^5	%	Mean x 10^5	%	Mean x 10^5	%
c-myc	-	0.7	45	1.2	80	2.3	78	2.5	91
	α			1.8	79	2.5	71	2.4	85
	γ			1.2	73	2.1	71	2.3	91
c-fos	-	1.7	60	1.2	48	2.7	63	2.5	71
	α			1.8	45	2.8	68	3.1	74
	γ			1.2	48	3.2	72	3.2	89

Table 7: Late change in *c-myc* and *c-fos* expression in response to BCGF and anti-μ in the HCs from patient 6: Effect of IFN. a) Levels remained constant up to this time.

Effects of γIFN. γIFN had no effect on the amount of the *c-myc* and *c-fos* proteins in HCs from 4 of the 5 patients studied (Tables 6 & 7). Whereas, in HCs from patient 4 γIFN caused a drop in both *c-myc* and *c-fos* protein expression comparable to that seen with αIFN (Table 5). However, this decrease in oncoprotein expression occurred later than the decrease caused by αIFN (at 24 as compared to 4 hours).

Effect of α and γIFN on oncoprotein levels in stimulated tonsil B cells

Treatment with BCGF and anti-μ caused an increase in the expression of both the *c-myc* and *c-fos* proteins within 4 hours of stimulation (Table 8). This increased oncoprotein expression remained constant throughout the period of culture, both in the presence and absence of α and γIFN (Table 8).

Effect of αIFN on *c-myc* and *c-fos* protein expression in Daudi cells

αIFN at 100U/ml had no effect on the expression of *c-myc* or *c-fos* during repeated testing during the first 24 hours of culture (Fig. 2). However, at 48 and 72 hours αIFN caused decreased expression of both *c-myc* and *c-fos* both in terms of the number of molecules per cell (data not shown) and the percentage of positive cells (Fig. 2).

Cell cycle

Cell cycle phases of HCL and CLL cells tested directly *ex vivo*. The great majority of purified B cells in HCL and CLL were in the G0/G1 phases of the cell cycle (Table 9). Slightly more S and G2/M cells were observed in HCL then in CLL cells.

In the patient who is resistant to αIFN treatment *in vivo* and whose cells proliferate *in vitro* in the absence of added growth factors (No. 7) more HCs were in the S and G2/M phases of the cycle than in the other HCL patients (Table 9) .

The high and low density tonsil fractions, representing resting and activated B cell populations, contained the expected low and high numbers of S phase cells (Table 9).

a. *c-myc*

Tonsil	IFN	0		4 hours		24 hours[a]	
		Mean x 10^5	%	Mean x 10^5	%	Mean x 10^5	%
1	-	<0.3	0.5	1.0	52	1.0	52
	α			0.9	73	0.9	59
	γ			1.2	69	1.1	79
2	-	0.9	48	1.2	60	1.2	46
	α			1.1	62	1.2	56
	γ			1.1	59	1.1	61

b. *c-fos*

Tonsil	IFN	0		4hrs		24hrs[a]	
		Mean x 10^5	%	Mean x 10^5	%	Mean x 10^5	%
1	-	1.2	35	4.3	55	4.4	68
	α			4.5	77	4.6	68
	γ			4.3	58	4.6	71
2	-	1.4	49	2.9	69	2.4	50
	α			2.7	59	2.3	59
	γ			2.3	68	2.1	68

Table 8: Effects of IFN on oncoprotein expression in tonsil B cells stimulated with BCGF and anti-μ. a) Levels remained constant throughout the remaining 6 days of culture.

Cell no.	Cell cycle phase		
	G0/G1	S	G2/M
HC 1	94	6	1
HC 2	99	1	0
HC 3	98	2	0
HC 4	98	2	0
HC 5	98	2	0
HC 6	95	5	0
HC 7	91	6	3
HC 15	95	3	2
HC 16	96	2	2
HC 17	95	3	2
HC 18	95	3	2
HC 19	95	4	1
Mean HC	95±2	4±2	1±1
CLL 1	98	2	0
CLL 2	99	1	0
CLL 3	99	1	0
CLL 4	98	2	0
Mean CLL	99±1	2±1	0
Tonsil 1[a]	89	11	0
Tonsil 2[a]	92	8	1
Tonsil 1[b]	75	25	0
Tonsil 2[b]	68	31	1

Table 9: Cell cycle analysis of purified B cells from HCL, CLL and tonsil tested directly *ex vivo.* a) High density fraction, b) Low density fraction.

Effects of αIFN on the cell cycle of HCs. During culture for 1 to 6 days without stimulation the cell cycle of HCs closely resembled that of cells tested directly *ex vivo* (Table 10). This was also true of the HCs from patient 7, even at 5 and 6 days when the cells were proliferating, as shown by the uptake of tritiated thymidine (Table 1).

HC no	αIFN	1 day			4 day[a]		
		G0/G1	S	G2/M	G0/G1	S	G2/M
1	-	98	2	0	93	8	0
	+	95	5	0	95	6	0
4	-	94	5	1	95	5	0
	+	93	7	0	94	6	1
5	-	99	1	0	95	5	0
	+	96	4	0	95	5	0
6	-	95	5	0	94	6	0
	+	93	8	0	95	5	0
7	-	97	3	0	96	4	0
	+	96	4	0	96	3	1

Table 10: Influence of αIFN on cell cycle phase of HCs. a) Levels remained constant between 1 and 4 days.

Effects of α and γIFN on the cell cycle phase of BCGF plus anti-μ stimulated HCs. Stimulated HCs from 3 patients (Nos. 1, 2, & 5) showed no change in the number of cells in each phase of the cell cycle throughout the 6 days of culture (Table 11). HCs from the other patients (Nos. 3 & 4) showed a small increase in the number of cells in the S and G2/M phases of the cell cycle at 1 and 5 days respectively (Table 11). Despite these differences in the number of cells in cycle all 5 patients HCs showed a similar incorporation of tritiated thymidine (Table 1).

In all 5 cases addition of αIFN to the culture had no effect on the cell cycle profile of the stimulated cells (data not shown) despite markedly reducing the proliferation as measured by the uptake of tritiated thymidine (Table 1).

γIFN treatment of HCs also had no effect on the proportion of stimulated cells in each phase of the cell cycle (data not shown), or on the incorporation of tritiated thymidine (Table 1).

HC no	Cycle phase	0 day	1 day	6 day[a]
1	G0/G1	94	98	98
	S	6	4	4
	G2/M	1	2	2
2	G0/G1	99	98	98
	S	1	2	2
	G2/M	0	0	0
3	G0/G1	98	90	90
	S	2	8	8
	G2/M	0	2	2
4	G0/G1	98	93	96
	S	2	7	4
	G2/M	0	0	0
5	G0/G1	98	98	94
	S	2	1	3
	G2/M	0	1	3

Table 11: Effect of BCGF and anti-μ on cell cycle phase of HCs. a) Levels remained constant between 1 and 6 days.

Effects of α and γIFN on the cell cycle of stimulated tonsil B cells. BCGF and anti-μ stimulation of tonsil B lymphocytes induced a small increase in the number of cells in the S phase of the cell cycle (Table 12), although, as with HCs, high levels of proliferation as indicated by the uptake of tritiated thymidine were seen (Table 1). α and γIFN had no effect on either the cell cycle profile (Table 12) or on the proliferation of the stimulated cells (Table 1).

Effect of αIFN on the cell cycle of Daudi lymphoblastoid cells. Daudi cells in contrast to HCs were proliferating with about 60% of cells in the S and G2/M phases of the cell cycle. αIFN had no effect on the number of dividing cells in the first 8 hours of culture. However, by 24 hours the number of cells in S and G2/M was halved (Fig. 4). Thereafter, for up to 72 hours, growth remained similarly arrested (Fig. 4).

Cell cycle	IFN	0	1 day	6 day[a]
G0/G1	-	95	89	89
	α		89	85
	γ		91	3
S	-	5	12	10
	α		11	15
	γ		11	15
G2/M	-	1	0	1
	α		1	0
	γ		0	0

Table 12: Effect of stimulation with and without α and γIFN on cell cycle phase of tonsil B lymphocytes. a) Levels remained constant between 1 and 6 days.

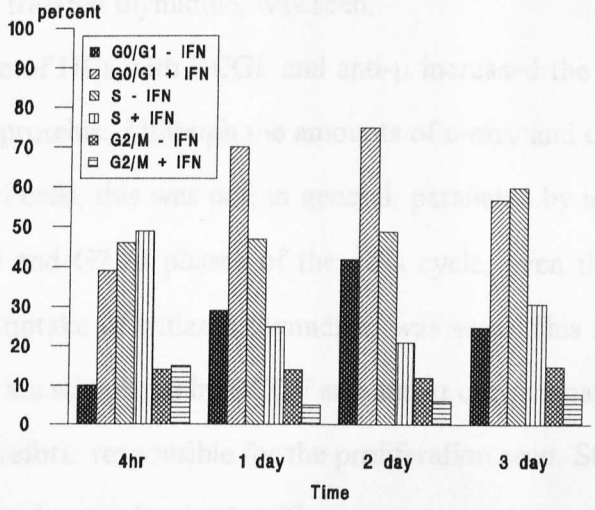


Fig. 4: Effect of αIFN on the cell cycle stage of Daudi cells.

DISCUSSION

HCs expressed variable amounts of the protein products of the oncogenes *c-myc* and *c-fos*. The levels of the *c-myc* protein in HCs were comparable to those found in both normal resting and CLL B lymphocytes, whereas the levels of *c-fos* were higher in HCs than in normal resting and CLL B lymphocytes, and, in general,

than in activated normal B lymphocytes. Since the levels of *c-myc* and *c-fos* are low and high respectively in differentiated non-dividing cells (Visser & Poppema 1990; Smeland *et al* 1985), the levels reported here reflect the state of HCs as differentiated cells which do not proliferate *in vitro*, and is in agreement with other studies on HCs (Lehn *et al* 1986; Pinto *et al* 1987).

αIFN had no effect on either *c-myc* or *c-fos* protein expression, or in the number of cells in cycle in unstimulated HCs. However, in the unusual case whose HCs proliferated *in vitro* in the absence of added growth factors, αIFN caused decreased *c-myc* and *c-fos* protein expression after 5 days of treatment. This decrease in oncoprotein expression was not paralleled by a decrease in the number of cells in the S and G2/M phases of the cell cycle, although reduced proliferation, as indicated by the uptake of tritiated thymidine, was seen.

Stimulation of HCs with BCGF and anti-μ increased the expression of both the *c-myc* and *c-fos* proteins. Although the amounts of *c-myc* and *c-fos* proteins increased in the majority of cells, this was not, in general, paralleled by increases in the number of HCs in the S and G2/M phases of the cells cycle, even though proliferation, as indicated by the uptake of tritiated thymidine, was seen. This indicates that although most of the HCs are stimulated by BCGF and anti-μ only a small number enter the cell cycle and are therefore responsible for the proliferation seen. Stimulation of normal B cells produced similar results, both with respect to oncoprotein expression and cell cycle phase, this correlates with previous reports (Smeland *et al* 1985; Munroe 1988).

Although αIFN altered the expression of the *c-myc* and *c-fos* proteins in HCs stimulated with BCGF and anti-μ, the time point of this change varied; in three patients αIFN delayed the increase in oncoprotein expression in response to stimulation. Such transient early changes following treatment are seen in other cell types (McNerney *et al* 1985; Conscience *et al* 1986). The decreased oncoprotein expression seen in the other patients HCs occurred at 5 days, and correlated with that seen in the patient whose cells undergo proliferation in the absence of added growth factors. The reason why HCs from different patients should respond at different time

points to the effects of αIFN on oncoprotein expression is unclear. *In vivo* treatment of HCs with αIFN results in a similar decrease in *c-fos* expression (Lehn *et al* 1986). In Daudi cells αIFN also caused a decrease in both the expression of *c-myc*, and the number of cells in cycle. This decrease in *c-myc* expression, which was followed by growth arrest, correlated with previous reports (Jonak & Knight 1984; Einat *et al* 1985; Resnitsky *et al* 1986). The decrease in *c-myc* and *c-fos* expression in HCs was followed by reduced proliferation, but not by a decrease in the number of cells in the S and G2/M phases of the cell cycle. This lack of correlation between tritiated thymidine incorporation and cell cycle, as measured by propidium iodine staining, has been noted before in Daudi cells where αIFN causes a 75% reduction in the incorporation of tritiated thymidine but only a 50% reduction in the number of cells in cycle (Schaffer *et al* 1988). In contrast to Daudi cells, where the majority of cells are in S and G2/M contributing to the incorporation of tritiated thymidine, only a small population of HCs are in cycle, hence any reduction in the number of HCs in cycle would be small and masked by the variation in the technique employed in the experiments reported in this chapter.

γIFN caused no changes in oncoprotein expression in BCGF and anti-μ stimulated HCs from four of the five patients examined, and a later change in *c-myc* expression in the HCs from the other patient. This indicated that the changes in oncoprotein expression induced by αIFN were largely specific to this cytokine. In addition, αIFN had no effect on normal B cells stimulated with these growth factors; indicating that the change in oncoprotein expression caused by αIFN was specific to the stage of B cell differentiation represented by the HC.

In conclusion, αIFN had no effect on either *c-myc* or *c-fos* protein expression on non-dividing HCs. Therefore, these results do not shed any light on the meaning of the phenotypic changes seen in Chapter 1. One might have expected an increase in the expression of both the *c-myc* and *c-fos* proteins if αIFN was inducing activation, conversely, if differentiation was occurring an increase in *c-fos* expression might be expected. However, αIFN did cause down regulation of both these proto-oncogenes

in proliferating HCs; one mechanism for the effect of αIFN in the treatment of HCL may therefore be its down regulation of *c-myc* and *c-fos* protein levels in the proliferating HC clone, the consequence of which would be a decreased rate of HC production.

THE ROLE OF TNFα AND ACCESSORY CELLS IN HC PROLIFERATION

CHAPTER 3

INTRODUCTION

As described in the previous chapter, αIFN treatment of HCs causes a reduction in the levels of the *c-myc* and *c-fos* proteins. In parallel with this reduced oncoprotein expression, HCs become unable to proliferate in response to stimulation with BCGF and anti-μ. αIFN has been shown to have this anti-proliferative effect on HCs regardless of the growth factor used to induce proliferation (Griffiths & Cawley 1990). Although HCs can be stimulated by a number of cytokines to produce a minor proliferative response *in vitro* (Griffiths & Cawley 1990; Barut *et al* 1990), the relevance of these cytokines to HC proliferation *in vivo* remains unknown. In view of the anti-proliferative effect of αIFN on HCs, it seemed important to establish whether the cytokine inhibits HC proliferation induced by physiological stimuli. The aim of this Chapter was to find which of the cytokines that stimulate HCs *in vitro* had any relevance to the proliferation of HCs *in vivo*, and to examine the effect of αIFN on the proliferation induced by these growth factors.

At the time work on this Chapter began, the cytokine TNFα had been recently reported to induce modest levels of HC proliferation (Cordingley *et al* 1988). In addition the cytokine was reported to induce an autocrine growth loop in HCs (Bianchi *et al* 1988). This, together with the fact that the levels of TNFα in the serum of HCL patients are raised (Foa *et al* 1990), suggested that TNFα might be responsible for the growth of HCs *in vivo*. However, the HCs used by Cordingley *et al* had only been crudely purified, leaving open the possibilities that TNFα was either not acting directly on the HCs but via contaminating accessory cells, or that the observed proliferation was not of HCs but of these contaminating cells. It therefore seemed important to show that TNFα could induce the proliferation of highly purified HCs. Therefore, for the experiments reported in the first part of this Chapter, myeloid precursor cells, which are found in increased numbers in HC blood (Michalevicz & Revel 1987), were completely removed from T-cell-depleted HCs prior to incubation

with TNFα. The results of these experiments indicate that TNFα does indeed directly induce HC proliferation.

It was also reported (Cordingley *et al* 1988) that repeated stimulation of HCs with TNFα could induce long term HC proliferation which eventually resulted in the formation of TNFα independent HC lines. Since such a continuous source of HCs would be a valuable research tool, it seemed important to follow up this work. Although HC lines were not produced, macrophages and adherent HCs formed close associations in the long term cultures; in fact, these macrophages may have been involved in inducing HC proliferation as they were associated with proliferating HCs. The details of this long term culture work form the second part of this Chapter.

At the same time as this association of HCs with macrophages in long term cultures was observed, independent work by a colleague in this Department showed that short term culture of monocytes/macrophages with HCs induced HC proliferation (Griffiths & Cawley 1991). Since HCs are frequently found in association with macrophages *in vivo* (Re *et al* 1988) these results raised the possibility that associations between HCs and accessory cells *in vivo* may result in the induction of HC proliferation. Other accessory cells which are found in association with HCs *in vivo* include endothelial cells in the spleen, liver and bone marrow (Ghadially & Skinnider 1982; Cawley & Burthem 1991), and fibroblasts which were thought to be associated with HCs because of the well known bone marrow fibrosis of HCL (Cawley 1980). (However, since the experiments reported in this Chapter began work in this laboratory has shown that it is not fibroblasts but HCs themselves which are responsible for this fibrosis (Burthem & Cawley 1993).) Investigation of the effects of these potential accessory cell types on HC proliferation, and the mechanisms involved in this proliferation, form the rest of this Chapter.

PATIENTS AND METHODS

Patients

PB HCs (9 cases) and splenic HCs (2 cases) were examined. All had entirely typical disease, except for 1 patient who was resistant to the effects of αIFN *in vivo* and whose HCs underwent proliferation in the absence of added growth factors *in vitro* (HC 7).

HC purification

PBM were isolated by density gradient centrifugation on lymphoprep. Splenic and tonsil tissue was dispersed though a wire mesh before centrifugation on lymphoprep (as in Chapter 1). T cells were removed prior to culture by one or two rounds of AET-treated SRBC rosetting, as required (as in Chapter 1).

Myeloid precursors and monocytes were removed using MAbs to CD14 (Leu M3) and CD34 (HPCA-1). (CD14 is a marker of mature monocytes, and CD34 is on myeloid precursor cells.) In addition, HCs were treated in parallel with class-specific controls (IgG1 and IgG2a) to ensure that depletion method used did not, per se, affect HC proliferation. MAbs and controls were diluted 1:10 and dialysed overnight against RPMI to remove azide. They were then filter sterilised before being incubated on ice for 30 minutes with T cell depleted HCs (1ml of MAb/2x10^7 cells). HCs were then washed twice, resuspended in RPMI containing sheep anti-mouse immunoglobulin coated beads (100μl/10^7 cells; Dynal), and incubated on ice for a further 40 minutes. The tube containing the cell suspension and magnetic beads was placed adjacent to a magnet for 3 minutes, and cells which had not bound via the MAb to the beads, and hence to the magnet, were carefully aspirated. This resulted in a pure population of HCs (CD19>99%, CD3<0.5%, CD14=0, CD34=0. For each antibody 2 x 10^5 cells were phenotyped by APAAP; for CD34 and CD14 the whole slide was examined and no positive cells were observed; n=4.)

Accessory cells

All cells were incubated at 37°C in 5% CO_2 in air.

Monocytes and macrophages. PBM were separated by density gradient centrifugation over lymphoprep. After washing $2x10^7$ PBM were incubated in 75ml tissue culture flasks, for 2 hours, in complete-RPMI, and washed thoroughly to remove any non-adherent cells. At this stage monocytes were either removed using 0.7% trypsin, or they were incubated for a further 5 days, by which time macrophage differentiation had taken place. Macrophages were then removed using 0.7% trypsin.

Human umbilical vein endothelial cells (HUVE). Umbilical cords were obtained from the Liverpool Maternity Hospital. Endothelial cells were stripped from the vein by filling it with 0.17% trypsin and incubating at 37°C for 8 minutes. The trypsin solution containing the endothelial cells was flushed from the vein, the cells were then washed, and HUVE were cultured to confluence in Iscoves modified minimal essential medium (IMEM) containing 20% new-born calf serum (NCS), 2mM L-glutamine 100U/ml penicillin, and 100µg/ml streptomycin (complete-IMEM). HUVE were used for culture with HCs at first passage.

Fibroblasts. A foreskin fibroblast line (FV1) was donated by Dr. Walker (Clatterbridge Hospital). FV1 cells were kept in continuous culture in complete-IMEM, and were subcultured with 0.7% trypsin when the cells had grown to confluence.

Epithelial cells. Two epithelial cell lines were used; SK.N.SH (EACC) was derived from intestinal epithelium and cultured in 50% Hams F12 and 50% MEMα containing 10% FCS, 2mM L-glutamine, 100U/ml penicillin and 100µg/ml streptomycin. INT 409 was derived from nervous epithelium and cultured in MEMα containing 10% FCS, 2mM L-glutamine, 100U/ml penicillin and 100µg/ml streptomycin. Cells from both lines were kept in continuous culture and subcultured as for fibroblasts.

Paraformaldehyde treatment. HUVE cells were plated out in 96 well plates and allowed to adhere overnight. The medium was removed from the adherent cells and

replaced with 1% paraformaldehyde, the cells were then incubated on ice for 2 hours. This time period had been shown to be the minimum required to kill the cells (data not shown). After 2 hours the paraformaldehyde was removed and the cells were washed thoroughly with PBS.

Membranes. HUVE cells were harvested and lysed for 20 minutes with 1mM Tris at 4°C, 0.5M Tris was then added to make an isotonic solution of 50mM Tris. Large cell debris was removed by centrifuging the lysed membranes at 2,000rpm for 10 minutes at 4°C. The supernatant containing the membranes was then pelleted at 50,000g for 1 hour at 4°C. The pellet was then resuspended such that there were membranes from $2x10^5$ HUVE/ml.

Matrix. HUVE were harvested, plated out at a concentration of $1x10^5$/ml in 24 well plates and allowed to adhere overnight. The cells were then treated with 0.5% Triton x 100 (Sigma) in PBS for about 5 minutes until they were observed to peel off. The plates were then thoroughly washed with PBS. In order to demonstrate that ECM was present the plates were fixed in methanol, and the matrix visualised using a MAb to fibronectin; staining with this MAb was positive (data not shown).

Measurement of proliferation

Thymidine incorporation. Proliferation was measured by adding either 0.5μCi (96 well plates) or 2.5μCi (24 well plates) of tritiated thymidine 18 hours before harvesting cells at 3, 6 and 9 days onto glass fibre filter papers. In order to ensure that all cells were harvested in the cultures containing accessory cells, the medium was carefully aspirated before adding trypsin to the cells for 5 minutes, the cells were then harvested as above. Incorporated tritiated thymidine was then measured by liquid scintillation counting (as in Chapter 2).

Proliferation was defined as CPM (counts per minute) in stimulated HCs being >1,500 and >3 times the level found in untreated HCs; these levels were chosen as counts >1,500 are rarely seen in the absence of stimulation, and as >3 times background is frequently used as a cut off for significant proliferation (Griffiths &

Cawley 1990). These values are to some extent arbitrary and may exclude low levels of proliferation.

Nuclear proliferation antigen staining. To ensure HCs were responsible for any proliferation observed Ki67 double staining was performed. Ki67 is a nuclear proliferation antigen which identifies cells in the S and G2/M phases of the cell cycle (Gerdes *et al* 1984). Cytospins were made from HCs collected at 3, 6 and 9 days. These were then stored at -20°C; slides from the day on which maximum proliferation occurred were then stained by APAAP for CD3, CD14, CD34, CD19 and light-chain sIg, plus class specific controls, together with HRP staining for Ki67 (Dako). Briefly, slides were fixed in acetone prior to incubating for 30 minutes with Ki67, followed by a 30 minute incubation with HRP-conjugated rabbit anti-mouse immunoglobulin. The HRP staining was visualised by incubation for 10 minutes with the DAB substrate; the nuclei of positive cells were stained brown. The slides were then incubated for 30 minutes with the second MAb, followed by 30 minutes with rabbit anti-mouse immunoglobulin and 30 minutes with APAAP; the later two steps were repeated with 10 minute incubations. The slides were then incubated with substrate for 20 minutes; the surface of positive cells was stained red, this staining did not appear to interfere with the brown nuclear Ki67 staining. The slides were then counterstained with haematoxylin (Appendix 3.1).

HC culture

For all the experiments reported in this Chapter HCs were cultured in complete-RPMI at a concentration of $2x10^5$ cells per well ($1x10^6$/ml) in 96 well plates, and $2x10^6$/well (ml) in 24 well plates.

Short term. HCs which had been either mock-depleted (with isotypic control MAbs), CD14 and CD34 depleted, or untreated, were all dealt with in the same way. HCs were incubated in the presence or absence of TNFα (10,000, 5000, 1000, 500 or 100U/ml), with and without the addition of αIFN or γIFN (1000 or 100U/ml).

Long term. HCs were cultured in 24 well plates in complete RPMI with 1000U/ml of TNFα for 12 days. At this time the medium was carefully aspirated and replaced by fresh complete-RPMI containing 100U/ml TNFα.

In order to ensure that any cell lines produced as a result of this long term culture were representative of HCs surface antigen staining was performed. Every 12 days the supernatant cells were removed and stained by indirect immunofluorescence for the following antigens CD3, CD4, CD19, CD20, CD23, CD25, light chain sIg, HC2, FMC7 and class I and II MHC, with the appropriate class specific controls, and then analysed by FACS (as in Chapter 1). In addition, the adherent cells from the same well were trypsinised, harvested, and the cells were used to make cytospins. The slides were then phenotyped by APAAP for the following antigens CD3, CD19, and CD68, together with HRP staining for Ki67 (as before). The cells were then phenotyped every 12 days until the cultures were exhausted.

Culture of HCs with accessory cells

Irradiation of accessory cells. HUVE and FV1 were harvested using trypsin and irradiated with 3,600rads, from a caesium-137 source; this dose was found to inhibit their proliferation while leaving them viable (Appendix 3.2). SK.N.SH and INT 409 cells required higher doses of irradiation 12,000rads, to achieve the same effect (Appendix 3.2).

Preparation of adherent layers. Irradiated cells were plated out, in the appropriate culture medium, at a concentration ($2x10^4$/well) which produced confluent monolayers once the cells had adhered. Cells were allowed to adhere overnight; cell debris and medium were then removed before adding HCs.

Culture conditions. HCs were added at a ratio of 10:1 adherent cell, and incubated in the presence or absence of TNFα with and without α or γIFN, all at 1000U/ml. TNFα induced proliferation was maximal at 1000U/ml (see Results); similarly, maximal inhibition of HC proliferation was seen with 1000U/ml αIFN (see

Results). Control wells of adherent cells and HCs alone were also set up with and without the addition of these cytokines.

The same culture conditions were employed for culture of HCs with paraformaldehyde treated HUVE and membranes from HUVE. Matrix was prepared in 24 well plates, but the same ratio of cells, and the same controls were used.

Titration of accessory cells. HUVE, monocytes, macrophages, FV1, SK.N.SH, INT 409 cells were incubated with the following ratios of accessory cells: HCs 1:10, 1:50, 1:100, 1:500 and 1:1000. However it was found that no proliferation of HCs was seen with any cell type at ratios <1:50, and with most cell types proliferation was absent or minimal at this ratio. Therefore, accessory cells were incubated with HCs at the following ratios 1:10, 1:15, 1:20, 1:30, 1:40 and 1:50.

Transwell plates. Transwell plates are 24 well tissue culture plates consisting of an outer well separated from an inner well by a membrane with 0.2μm pores (Fig. 1). This allows the free passage of nutrients, growth factors, etc. between the two cell types without cell contact. HUVE cells were harvested, irradiated, placed in the outer wells and allowed to adhere overnight as before. HCs were then placed in the inner well at a ratio of 10:1 to adherent cells (Fig. 1). The following control cultures were also set up; HUVE in contact with HCs, HUVE only, and HCs only.

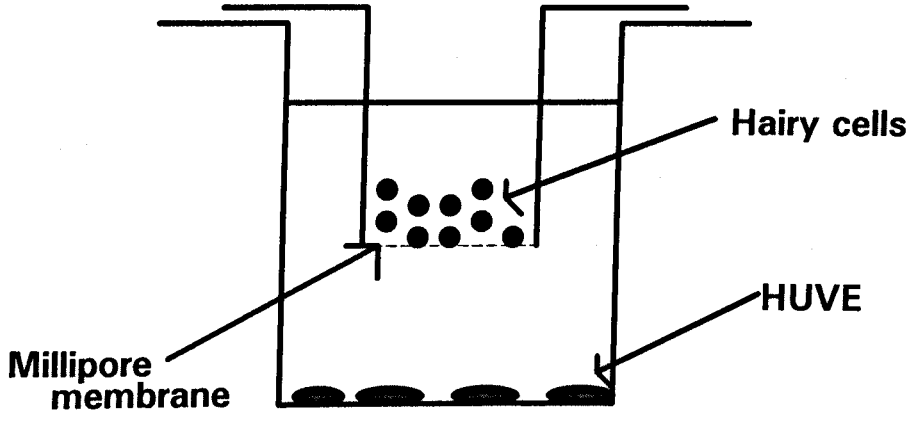


Fig. 1: Culture of HCs with HUVE in transwell plates

Culture supernatants and cytokines

Preparation of culture supernatants. The culture medium was removed from flasks of HUVE that had been allowed to grow to confluence, the medium was then filtered through a 0.2μm membrane to remove any cells or cell debris. The concentration of this neat culture supernatant was regarded as 100%, and other percentages quoted are in reference to this.

Incubation of supernatants and cytokines with HCs. HCs were incubated at a concentration of $2x10^5$/well in 96 well plates together with the following culture supernatants/cytokines (concentrations); HUVE (50%), 5637 (10%), GM-CSF (1000U/ml), G-CSF (100U/ml), IL-1β (50U/ml), IL-6 (50U/ml) or IL-1β plus IL-6. GM-CSF, G-CSF, IL-1β and IL-6 were used since the accessory cells which induce HC proliferation all produce these cytokines (Mantovani *et al* 1989; Seiffer *et al* 1987); 5637 culture medium also contains the same range of cytokines (Kohase *et al* 1987; Seiff *et al* 1987).

Concentration of supernatants. As supernatant from HUVE (50%) induced HC proliferation that was less than when the cells were in direct contact, the effect of increasing the concentration of supernatant was examined. Supernatant from HUVE cells was concentrated 10 fold by Amicon ultrafiltration. Briefly, HUVE were grown to confluence and then cultured for 24 hours in 1% NCS, the supernatant was then removed and passed through a 30,000 molecular weight filter using pressure from nitrogen gas. (Medium containing 1%, rather than 20%, NCS was used since the levels of HC proliferation were found to decrease as the concentration of medium containing 20% NCS increased.) These concentrated supernatants were then filtered through a 0.2μm filter and frozen at -20°C prior to use. Supernatants were then incubated at concentrations of 200, 100 and 50% with HCs. Maximum proliferation was seen with supernatants at 200% and this concentration was used for subsequent experiments.

MAb inhibition of supernatant cytokines. MAbs to TNFα (Seralab), together with GM-CSF and IL-1β (The Binding Site), were used. The MAb to TNFα was used

since, of the growth factors known to be produced by the accessory cells, only TNFα had any effect on HC proliferation (see Results); MAbs to GM-CSF and IL-1β were used as controls to show than inhibition of irrelevant cytokines did not effect HC proliferation. MAbs to TNFα, GM-CSF, and IL-1β were then incubated at 4.8, 56 and 12.3μg/ml respectively, with HUVE supernatants, or cells, for 30 minutes at 4°C, before being cultured with HCs. These anti-cytokine MAbs were titrated and the doses used maximally inhibited the effects of their respective recombinant cytokines in appropriate assays.

Adhesion assays

Measurement of adhesion. HUVE were plated out at $1x10^4$ cells/well in 96 well plates and allowed to grow to confluence. Medium was then removed from the cells and $2x10^5$ HCs were added per well and incubated at 37°C for the following times; 15 and 30 minutes, 1, 2, 4 and 6 hours. Adherence was then measured using the Rose Bengal Method (Lopez *et al* 1984). Briefly, adherent HCs were fixed using 2% gluteraldehyde, non-adherent HCs were then carefully aspirated, and the plates washed twice with warm complete-RPMI. Cells were then incubated with 0.25% Rose Bengal for 10 minutes, the stain was then removed from the plates which were washed 4 times with PBS before visualising the stain with 100μl of 50% ethanol in PBS. Absorbance was measured at 570nm; the amount of absorbance being proportional to the number of adherent cells. It was found that HC adherence was maximal after 2 hours incubation.

Inhibition of adhesion. MAbs to the following antigens were used (concentrations μg/ml) CD11a (5, 10 & 20), CD11b (5, 10 & 20), CD11c (5, 10 & 20), CD18 (20, 35 & 50) and CD54 (10, 18 & 25), with IgG1, IgG2a and IgG2b as class specific controls. MAbs to the leukocyte integrins were chosen since there was no evidence that binding of HCs to ECM had any effect on their proliferation (see Results), although HCs posses antigens that can mediate this binding. The concentrations of these MAbs used covered the ranges over which they had

previously been shown to inhibit adhesion (Kishimoto *et al* 1989; Bevilacqua *et al* 1987; Schleiffenbaum *et al* 1989; Loike *et al* 1991; Keizer *et al* 1987; Schlesinger *et al* 1991: Luschinskas *et al* 1991). HCs ($2x10^5$/well) were incubated with these MAbs for 30 minutes prior to adding HUVE and performing the adhesion assay, as above. Significant inhibition of adhesion was seen with MAbs against the CD11b, CD11c, CD18 and CD54; this inhibition was maximal at 20, 5, 20 and 18μg/ml respectively (Table 1).

MAb	Conc.[a] μg/ml	Bound HC (%)
None	-	100±10
IgG1	50	83±10
IgG2a	20	58±10
IgG2b	25	67±10
CD11a	20	58±10
CD11b	20	27±8
CD11c	5	27±8
CD18	20	25±5
CD54	18	17±3

Table 1: Inhibition of HC binding to HUVE by MAbs to adhesion molecules. a) Conc. = concentration. b) Mean ± SEM; n=2.

Having shown that these MAbs inhibited adhesion of HCs to HUVE their effect on HUVE mediated HC proliferation was examined. HUVE were irradiated, plated out in 96 well plates, and allowed to adhere overnight. HCs were then incubated for 30 minutes with the appropriate concentrations of MAbs to CD11a, CD11b, CD11c, CD18 and CD54, together with IgG1, IgG2a and IgG2b as class specific controls prior to culture for up to 9 days with HUVE.

RESULTS

TNFα stimulation of highly purified HCs

TNFα caused proliferation of T cell depleted HCs in 4 of the 5 cases studied (Table 2). Maximum proliferation was seen at 1000U/ml of TNFα (data not shown). Neither mock depletion with class specific control antibodies nor depletion of myeloid precursors and monocytes with CD34 and CD14 had any effect on the levels of proliferation of these T-depleted HCs (Table 2). HCs were responsible for this proliferation as all the Ki67 positive cells were both light-chain sIg restricted and CD19 positive.

HC no.	TNFα[a]	Depln.[b]	6 day[c]	9 day[c]	Ki67[d]
2	-	-	1153±75	300±94	0.1
	+	-	3678±618	1401±126	ND
	+	+	5066±705	1544±119	1.5
3	-	-	1084±184	2379±42	0
	+	-	2069±191	1498±115	ND
	+	+	401±28	1936±145	2
3s	-	-	841±123	1857±55	ND
	+	-	2900±149	3927±390	ND
	+	+	2108±179	2554±346	ND
4	-	-	191±9	178±35	0.5
	+	-	900±113	3010±536	ND
	+	+	4219±968	3624±275	2
11	-	-	240±63	785±92	0.2
	+	-	1006±35	2369±207	ND
	+	+	912±147	2206±44	2

Table 2: Effect of TNFα on proliferation of T-cell depleted HCs with and without the removal of CD14 and CD34 cells. a) 1000U/ml TNFα. b) Depln.=depletion of CD34 & CD14. c) CPM ± SEM. d) 9 day, all Ki67 +ve cells CD19 +ve, light-chain sIg restricted.

Both 100 and 1000U/ml αIFN caused reduced HC proliferation in all cases, however this reduction was greatest at 1000U/ml (Table 3); whereas the effects of γIFN varied (data not shown).

HC no.	αIFN[a]	6 day[b]	9 day[b]
2	-	3678±618	1401±126
	+	1476±618	1294±712
3	-	2069±191	1498±115
	+	570±103	320±30
3s	-	2900±149	3927±390
	+	585±207	1768±142
4	-	900±113	3010±536
	+	254±24	844±179
11	-	1006±35	2369±207
	+	598±53	1191±83

Table 3: Effect of αIFN on TNFα induced HC proliferation. a) αIFN at 1000u/ml. b) CPM±CPM. s=splenic HCs.

Effect of long-term exposure of HCs to TNFα

Repeated stimulation of HCs every 12 days with 1000U/ml of TNFα induced long term HC survival, with the absolute number of viable cells remaining constant, while in the control cultures, which lacked TNFα, there were no viable cells after 12 days. The maximum period that HCs could be sustained in the presence of TNFα was 48 days; by 60 days no viable cells remained.

As a result of this repeated stimulation with TNFα a number of phenotypic and morphological changes occurred in the cultures. The most marked of these changes was the appearance of patches of adherent cells (Fig. 2a). At the beginning of culture these cells were mainly HCs (CD19 positive, light-chain sIg restricted), with a small

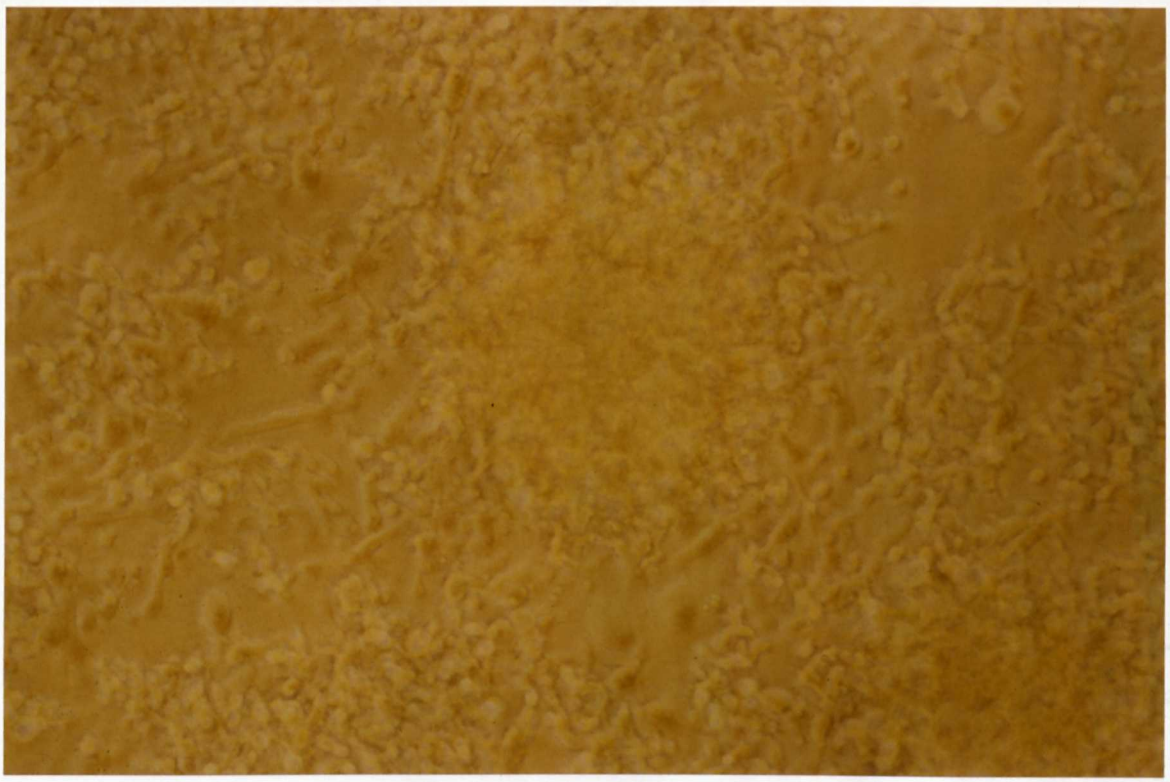
number of macrophages (CD68 positive cells; data not shown). With time the number of macrophages in these patches increased markedly, while the relative number of HCs decreased (data not shown). Both HCs and macrophages in these patches were shown to be undergoing proliferation as shown by the presence of the nuclear proliferation antigen Ki67 (3%; n=4). Clumps of HC were located above these patches, whereas the majority of other supernatant cells were single cells in suspension (Fig. 2b).

In order to establish if any lines formed were representative of HCs the phenotype of supernatant HCs was also examined. Although all the supernatant cells remained light-chain sIg restricted, CD19 positive, 2 populations of B cells appeared after 12 days in culture; these were differentiated by both size (Fig. 3a) and surface phenotype (Fig. 3b). The population of larger cells (approximately 25% of the cells) had a higher expression, as measured by modal fluorescence, of CD19, light-chain sIg, FMC7 and class I and II MHC, together with all the detectable expression of CD25 and HC2 (Fig. 3b). It is suggested that these larger cells represent activated cells. Whether or not these cells were proliferating could not be determined since, at the time, the Department had no access to a FACS cell sorter.

The effect of different accessory cells on HC proliferation

Endothelial cells and fibroblasts. Both HUVE (4/6 cases) and FV1 (4/6 cases) induced HCs to proliferate (Table 4). The effect of endogenous monocytes in the patient whose HCs proliferated in the absence of added growth factors (HC 7) could be replaced by both HUVE and FV1 cells (Table 4). All the Ki67 positive proliferating cells were HCs (light-chain sIg restricted, CD19 positive cells; data not shown).

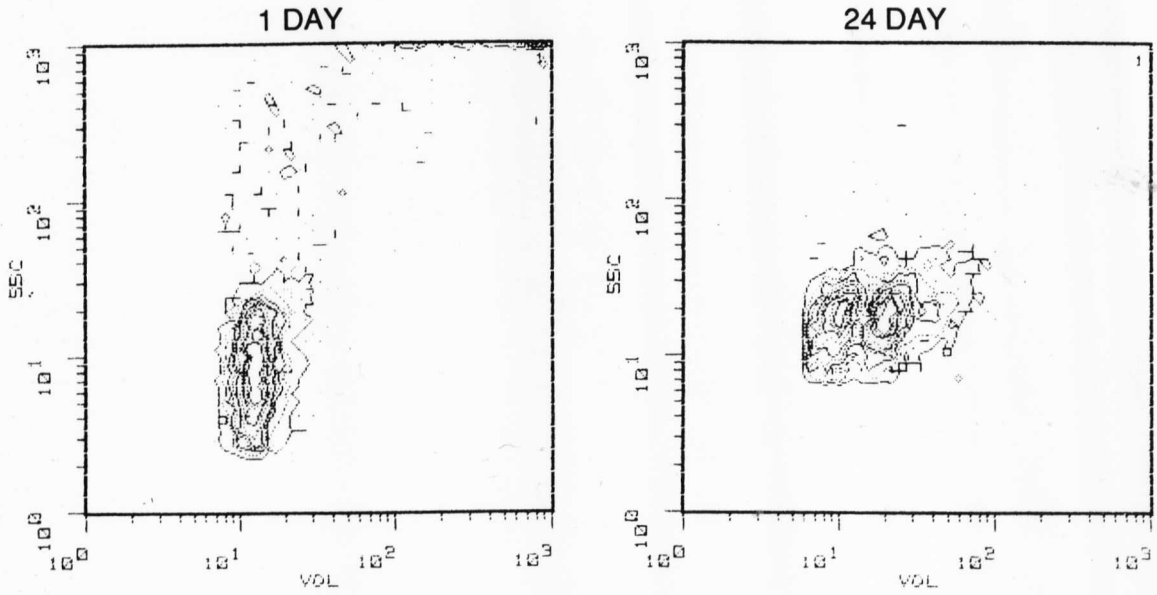
a) Adherent cells with clumps of HCs



b) Single supernatant HCs

Fig. 2: Morphology of HCs in long term culture with TNFα.

a) Cell size



b) Phenotype

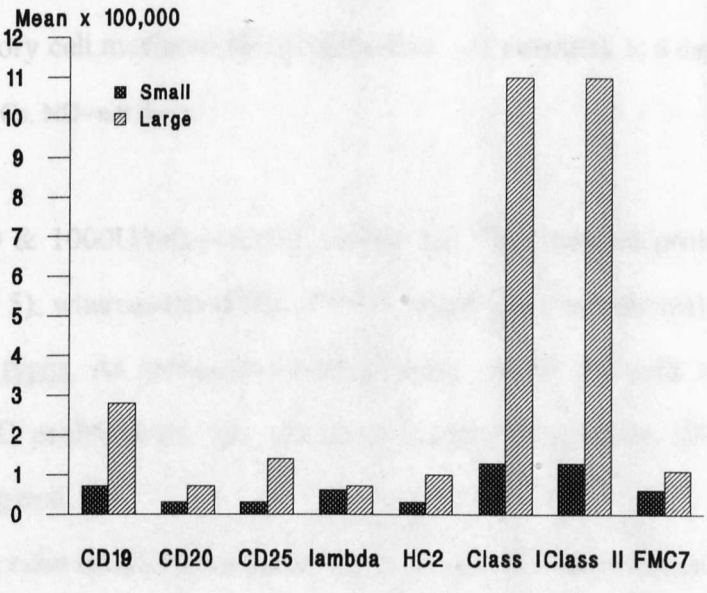


Fig 3: HC populations found in the supernatants of long term cultures with TNFα.

(One representative case at 12 days; similar results were obtained at later time points).

HC no.	Accessory cell	6 day[a]	9 day[a]	Ki67[b]
2	-	345±56	218±48	ND
	HUVE	386±142	106±28	0.5
	FV1	1683±198	2072±133	5
3s	-	2172±267	8587±1982	ND
	HUVE	18,249±3185	24,001±6300	10
	FV1	22,457±213	23,184±3401	9
4	-	79±17	111±50	ND
	HUVE	613±134	832±82	ND
	FV1	304±22	362±91	ND
7[c]	-	121±39	622±494	ND
	HUVE	22,683±781	16,482±3506	24
	FV1	15,640±966	13,222±900	15
9	-	738±276	808±239	ND
	HUVE	5373±687	2674±334	7
	FV1	1798±457	2014±708	4
11	-	303±70	564±233	ND
	HUVE	26,232±2148	11,590±1245	21
	FV1	5300±233	9631±2723	2

Table 4: Accessory cell mediated HC proliferation. a) CPM±SEM. b) 6 day value. c) CD14 & CD34 depleted HCs. ND=not done.

αIFN (100 & 1000U/ml) reduced HUVE and FV1 induced proliferation in all cases (4/4; Table 5); whereas the effect of γIFN varied (data not shown).

Other cell types. As monocytes/macrophages, endothelial cells and fibroblasts all stimulated HC proliferation, the effects of other cell types on HC proliferation were also investigated.

At the high ratio of HCs to accessory cells used (10:1) both intestinal (INT 409) and nervous tissue (SK.N.SH) epithelial cell lines could mediate HC proliferation in 4/4 and 1/4 cases respectively (Table 6). As with HUVE and FV1, TNFα had variable

effects on the induced proliferation, whereas αIFN was always inhibitory (data not shown).

HC no.	αIFN[a]	6 day[b]	9 day[b]
3s	-	18,249±3185	24,001±6300
	+	9,577±712	21,259±1133
7[c]	-	22,683±781	16,482±3506
	+	11,948±1058	13,376±796
9	-	5373±687	2764±334
	+	295±42	153±59
11	-	26,232±2148	11,590±1245
	+	2886±357	6510±513

Table 5: Effect of αIFN on HC proliferation induced by HUVE. a) αIFN at 1000U/ml. b) CPM±SEM. c) CD14 & CD34 depleted HCs.

HC no.	Cell type	6 day[a]	9 day[a]
2	-	206±78	294±89
	INT 409	1738±168	658±131
	SK.N.SH	159±7	125±39
3s	-	1924±218	1127±120
	INT 409	5729±1047	4023±1150
	SK.N.SH	2445±133	2071±185
9	-	316±80	949±200
	INT 409	12,559±1184	3584±89
	SK.N.SH	1002±79	633±140
11	-	398±25	1900±140
	INT 409	1624±352	4434±505
	SK.N.SH	3700±529	13,146±2909

Table 6: Epithelial cell mediated HC proliferation. a) CPM±SEM.

Effect on HC proliferation of different ratios of accessory cells:HCs

In order to establish the relative ability of the different accessory cell types to induce HC proliferation, the proliferative experiments were carried out at different accessory cell: HC ratios.

Proliferation was seen with all the accessory cell types at the highest ratio, 1 accessory cell to 10 HCs (HUVE (4/4 cases), FV1 (3/4 cases), macrophages (2/4 cases), monocytes (3/4 cases), SK.N.SH and INT 409 (both 1/4 cases). (The HCs used for these experiments were not from the same patients as those described earlier). At ratios of <1:20 only macrophages (2/4 cases) and endothelial cells (4/4 cases) still induced HC proliferation. As the ratio of accessory cells to HCs decreased the amount of proliferation also decreased, and, at ratios of >40 HCs to each accessory cell, only HUVE (4/4 cases) still induced HC proliferation (representative case, Fig. 4). Hence HUVE cells consistently stimulated higher levels of HC proliferation than any of the other cell types tested.

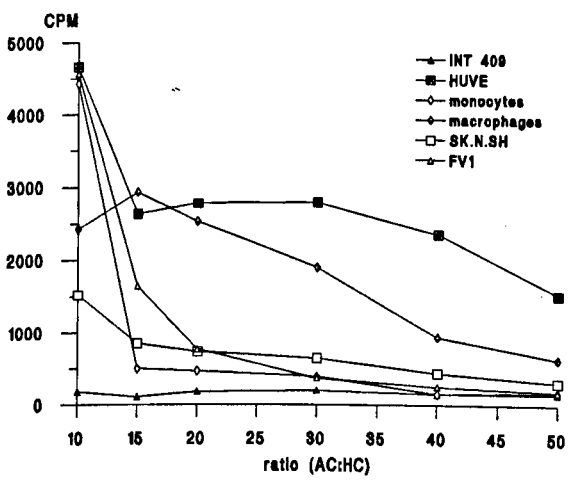


Fig. 4: Effect of different ratios of HCs:accessory cells in HC proliferation (1 representative case). INT 409 stimulated HC proliferation in another case.

Analysis of the mechanism of accessory cell mediated proliferation

As HUVE cells were the most potent mediators of HC proliferation they were used to analyse the mechanisms of this accessory cell mediated proliferation.

Are viable cells needed? Fixation of HUVE with paraformaldehyde abrogated the accessory cell mediated proliferation in all 3 cases studied (Table 6). This abrogation was not due to a residual cytotoxic effect of the paraformaldehyde, as TNFα could induce HC proliferation in the presence of paraformaldehyde-treated HUVE (3/4 cases; Table 7).

HC no.	PFH[a]	TNFα	6 day[b]	9 day[b]
3	-	-	22,869±4520	30,440±5420
	+	-	109±83	2378±124
	+	+	49±16	12,678±124
7[c]	-	-	41,651±1269	6222±2213
	+	-	20±7	40±15
	+	+	32±11[d]	22±5[d]
11	-	-	2466±1925	28,900±287
	+	-	27±0	844±65
	+	+	210±9	2558±213

Table 7: Effect of fixation on HUVE mediated HC proliferation. a) Paraformaldehyde. b) CPM±SEM. c) CD34 & CD14 depleted HCs. d) HCs from this patient did not proliferate in response to TNFα.

Can membranes substitute for viable cells? HCs did not proliferate in the presence of HUVE membranes (3/3 cases; data not shown): intact HUVE did induce HC proliferation in these 3 cases. TNFα did not synergise with HUVE membranes to cause increased HC proliferation above that seen with the cytokine alone (data not shown).

Can matrix substitute for viable cells? No proliferation was seen when HCs (2/2 cases) were incubated with ECM from HUVE (data not shown); although matrix components were shown to be present by staining with a MAb to fibronectin. As before, intact HUVE induced HC proliferation in both these cases. The lack of proliferation was not due to a residual effect of the treatment used to remove the

adherent cells as TNFα induced HC proliferation in the presence of this matrix (data not shown); in addition, TNFα did not synergise with the matrix components to induce HC proliferation above that seen with TNFα alone.

Is cell contact necessary? Proliferation of HCs when physically separated from HUVE by a millipore membrane in transwell plates was markedly less than when the two cell types were in contact (4/4 cases; Table 8).

HC no.	HUVE	TW[a]	3 day[b]	6 day[b]
2	-	+	595±20	743±94
	+	-	3592±167	7112±611
	+	+	1061±153	2795±311
3s	-	+	1397±20	1864±149
	+	-	3535±163	1730±117
	+	+	488±91	579±129
10	-	+	2486±162	1544±10
	+	-	18,472±338	8460±435
	+	+	2203±465	378±60
11	-	+	222±35	166±39
	+	-	26,510±3238	29,186±8532
	+	+	2839±633	3736±450

Table 8: Effect of transwell separation of HCs from accessory cells. a) TW=transwells. b) CPM±SEM.

HUVE supernatants at 50% induced HC proliferation in all 4 cases (data not shown). Increasing the concentration of this supernatant resulted in higher levels of proliferation (data not shown), and this was maximal at 200% (Table 9). In addition, 5637 culture supernatant produced a similar level of proliferation to HUVE supernatant (data not shown). Proliferation was induced by TNFα (4/4 cases), but not by any of the other cytokines known to be produced by HUVE viz. IL-1β, IL-6, G-CSF or GM-CSF (data not shown). The amount of HC proliferation induced by

TNFα was always less than that induced by intact HUVE (data not shown); whereas the proliferation induced by HUVE supernatant was often of the same order of magnitude as that induced by intact HUVE (Table 9)

HC no.	HUVE	MAb to TNFα	6 day[a]	9 day[a]
12	-	-	294±26	428±57
	cells	-	4325±867	2842±404
	"	+	4532±850	4123±1572
	supt.[b]	-	419±18	3074±74
	"	+	396±80	770±43
12s	-	-	417±39	637±70
	cells	-	1111±35	2910±242
	"	+	ND	ND
	supt.	-	569±26	1990±193
	"	+	57±11	154±6
13	-	-	550±51	1402±119
	cells	-	2418±41	4932±246
	"	+	ND	ND
	supt.	-	1111±51	4044±272
	"	+	41±4	41±11
14	-	-	349±79	710±148
	cells	-	14,515±755	12,713±321
	"	+	13,502±927	11,492±193
	supt.	-	336±42	2402±449
	"	+	415±52	701±59

Table 9: Effect of MAb to TNFα on HUVE induced HC proliferation. a)CPM±SEM. b) Supt=supernatant at 200%

From these results it appears that both factor(s) in the supernatants of accessory cells, and contact with these cells were necessary to induce HC proliferation. The nature of the molecules involved in both these effects was therefore investigated.

Nature of the supernatant effect

The MAb to TNFα abrogated HC proliferation induced by HUVE supernatants (Table 9); in contrast the MAbs to IL-1β and GM-CSF had no effect (4/4 cases; data not shown). This indicated that TNFα is necessary for the induction of HC proliferation induced by HUVE supernatant. However inhibition of TNFα did not affect the proliferation of HCs in response to HUVE cells (Table 9).

Nature of the contact effect

Adhesion of HCs to HUVE was inhibited by MAbs to CD11b, CD11c, CD18 and CD54 (Table 1); whereas neither the MAb to CD11a, or isotypic controls had any significant effect on the number of adherent HCs (Table 1).

HUVE-mediated HC proliferation was partially inhibited by MAbs to CD11b, CD11c, CD18 and CD54 (Table 10). In contrast, no inhibition of proliferation was seen with either the isotypic control MAbs (data not shown) or the MAb to CD11a (Table 10).

DISCUSSION

The results in this Chapter clearly indicate that TNFα is indeed a proliferative signal for HCs. The presence of the nuclear proliferation antigen, Ki67, on HCs stimulated with TNFα indicated that these cells were responsible for the proliferation observed. In addition, TNFα was shown to be directly inducing HC proliferation, as removal of possible accessory cells (myeloid precursor cells and T-cells) did not alter the levels of proliferation observed. These results indicate that the previously reported proliferation of HC PBM in response to TNFα (Cordingley *et al* 1988) was indeed due to the cytokine directly inducing HC proliferation. In this respect HCs respond in the same way to TNFα as normal activated B lymphocytes which proliferate in response to the cytokine both *in vivo* (Zola & Nikoloutsopoulos 1989) and *in vitro* (Kerl *et al* 1989).

HC no.	MAb	6 day[a]	9 day[a]
12	-	3575±397	6105±791
	CD11a	3837±264	5778±498
	CD11b	2505±159	2817±259
	CD11c	2560±271	2852±171
	CD18	1975±471	1852±283
	CD54	2582±218	3529±382
12s	-	1111±35	2910±242
	CD11a	1067±106	2619±143
	CD11b	895±102	1255±152
	CD11c	851±108	1083±174
	CD18	731±50	1098±99
	CD54	838±104	1021±109
13	-	2418±41	4932±246
	CD11a	2582±386	5186±426
	CD11b	870±46	2710±107
	CD11c	1028±10	770±1031
	CD18	1194±230	2794±65
	CD54	2357±241	2643±271
14	-	3274±96	13,322±2579
	CD11a	3893±567	13,512±1983
	CD11b	4412±220	9383±460
	CD11c	4708±818	8807±343
	CD18	4524±29	9726±576
	CD54	4487±170	9946±167

Table 10: Inhibition of HUVE mediated proliferation by MAbs to adhesion molecules. a) CPM±SEM.

Long term culture of HC PBM with TNFα resulted in the appearance of adherent patches of HCs which, with time, became interspersed with an increasing number of macrophages. Staining with the nuclear proliferation antigen Ki67 indicated that both the macrophages and HCs in these patches were proliferating. Since virtually no monocytes were present in the initial cell preparations, these macrophages presumably appeared as a result of TNFα inducing the differentiation of the myeloid

precursors which are found in HCL PBM (Michalevicz & Revel 1989). Clumps of supernatant cells were consistently seen to be attached to these patches. These findings, taken together with parallel independent work in the Department, suggested that macrophages could stimulate HC proliferation *in vitro* (Griffiths & Cawley 1991), and raised the possibility that interactions between HCs and macrophages might be of pathophysiological importance in HCL. Furthermore, since HCs are known to form close associations with endothelial cells in the spleen and liver (and perhaps with fibroblasts in the marrow), these findings prompted a more general examination of the effects of accessory cells on HC proliferation.

A range of possible accessory cell types was examined and, although all could potentially stimulate HC proliferation at very high accessory cell:HC ratios, only endothelial cells, and to a lesser extent macrophages, could stimulate proliferation at ratios <1:15.

The fact that endothelial cells supply the most stimulatory micro-environment for HC proliferation *in vitro* may provide a possible explanation of the unique distribution of HCs *in vivo* to the spleen and liver which are rich in these cells (Jansen *et al* 1984), and may explain why removal of the spleen in HC patients causes alleviation of the disease.

Having shown that the endothelial cell micro-environment provided the most potent proliferative signal to HCs, the mechanisms involved in this proliferation were investigated. In order to do this the relative roles of cell contact and soluble factors were investigated

Both HUVE physically separated from HCs and HUVE supernatants induced HC proliferation. However, the levels of this proliferation were generally less than when the cells were in direct contact. The supernatant effect was shown to be mediated by TNFα, since proliferation induced by HUVE supernatants was inhibited by a blocking MAb to this cytokine. However, the MAb did not inhibit the proliferation of HCs induced by HUVE themselves, indicating that in addition to

being present in the supernatant the cytokine passes directly between the closely bound cells.

Contact between HUVE and HCs did not, per se, induce HC proliferation. Thus HCs did not proliferate in response to paraformaldehyde-treated HUVE, to HUVE membranes or to HUVE-produced ECM. However, the fact that physical separation of HUVE from HCs induced lower levels of proliferation than when the cells were in direct contact indicated that binding of HCs to viable HUVE was important. The molecules involved in this adhesion were therefore investigated. Binding was partially inhibited by MAbs to CD11b, CD11c, CD18 and CD54 indicating that adhesion is mediated through binding of CD11b/CD18 and CD11c/CD18 on HCs to CD54 and the CD11c ligand (which is unknown) on HUVE. In addition to inhibiting HUVE binding to HCs, MAbs to CD11b, CD11c, CD18 and CD54 also partially inhibited HC proliferation. These findings confirm that cell contact plays a role in accessory cell-mediated proliferation and that this cell contact is mediated by HC leukocyte integrins. These results do not indicate whether these molecules are directly involved in producing the proliferative signal or whether they enable the cells to form sufficiently close contact for signalling to occur via other molecules. In conclusion then, the present findings indicate that a co-operative stimulus involving TNFα and HC leukocyte integrins is necessary for optimal HC proliferation.

The growth and differentiation of normal B lymphocytes involves similar associations between the haemopoietic cells and the accessory cells of the stromal cell layer (Whitlock & Witte 1982). Both adherence to, and factors produced by, stromal cells are vital for the maintenance of B lymphocyte growth and differentiation in these cultures (Kincade *et al* 1987). Pre-B lymphocytes adhere to fibronectin in the ECM produced by the cells of the bone marrow stroma (Roldan *et al* 1992). As in HC accessory cell cultures this adherence does not, on its own, induce the proliferation of these pre-B lymphocytes, however it does increase the proliferation of these cells in response to stromal cell culture supernatants (Lemoine *et al* 1990). As with HCs, this proliferation involves the accessory cells binding to more than one adhesion molecule;

the pre-B cells also bind via CD44. In addition, growth factors are involved with different factors (stem cell factor, IL-7, and IL-6) acting primarily on different stages of the B lymphocyte lineage (Funk & Witte 1992; Takai *et al* 1992; Roldan *et al* 1991). The growth and differentiation of more mature B lymphocytes in response to other cell types is also mediated by both adherence and growth factors. The proliferation of mature B cells in response to follicular dendritic cells (FDCs) in the lymph nodes is dependent on both growth factors (IL-6) and adhesion via CD54 binding to CD11a/CD18 (Kosco *et al* 1992).

αIFN reduced HC proliferation induced by both TNFα and accessory cells. Hence, *in vivo* treatment of HCL with αIFN would reduce the HC proliferation induced by accessory cells, possibly via the reduced expression of the *c-myc* and *c-fos* proto-oncogenes, as reported in Chapter 2. This would result in reduced tumour burden, as is seen with αIFN treatment *in vivo*.

Finally, the results of this Chapter assign pathophysiological importance to the HC-endothelial cell and HC-macrophage associations long known to occur in the spleen and liver respectively.

C-FMS PROTEIN EXPRESSION BY HCS

CHAPTER 4

INTRODUCTION

The *c-fms* proto-oncogene encodes the receptor for the macrophage colony stimulating factor (M-CSF) (Sherr *et al* 1985); the gene product is a transmembrane glycoprotein with a molecular weight of 150kD (Sherr 1990). *C-fms* expression is mainly restricted to the cells of the monocyte/macrophage lineage (Ashmun *et al* 1989; Rettenmier *et al* 1986) and to the placenta and its associated tumours (Arceci *et al* 1989; Kacini *et al* 1990). The amount of the *c-fms* protein on the cells of the monocyte/macrophage lineage increases with the maturity of the cell (Sariban *et al* 1985).

Binding of M-CSF to the *c-fms* protein causes phosphorylation of the later molecule on tyrosine (Yeung *et al* 1987; Tapley *et al* 1990). This is followed by internalisation of the receptor ligand complex (Tamura *et al* 1989), resulting in a rapid and lasting decrease in the expression of the *c-fms* protein at the cell surface (Rettenmier *et al* 1987). Expression of the *c-fms* protein is also down regulated by other cytokines, such as TNFα (Sherr 1989), and by PMA (Sariban *et al* 1989); whereas GM-CSF has no effect (Sherman *et al* 1990). Transfection of cells with the *c-fms* gene enables them to respond to M-CSF by undergoing proliferation together with the morphological and phenotypic changes normally associated with myeloid differentiation (Pierce *et al* 1990; Rohrschneider & Metcalf 1989).

The effect of M-CSF on monocytes/macrophages varies depending on their stage of maturation: in more immature cells the cytokine causes the morphological (Wilman *et al* 1989; Munn *et al* 1990) and immunophenotypic (Pierce *et al* 1990; Geisler *et al* 1989) changes associated with differentiation. However, M-CSF causes the proliferation (Hume *et al* 1988; Sherr 1988) and increased survival (Munn *et al* 1990) of cells throughout the monocyte/macrophage lineage.

Although HCs are B cells they have a number of features in common with cells of the monocyte/macrophage lineage (Braylan *et al* 1978; Falini *et al* 1985). In view of these monocytoid features it was decided to examine the *c-fms* expression of HCs. It was hoped that this would add further information to that discussed in Chapter 3,

on the factors involved in the pathogenesis of HCL. In this Chapter it is shown that, among B cells, the *c-fms* protein is specifically expressed by HCs, and by a population of activated normal B cells. Some possible effects of M-CSF on HCs are then examined.

PATIENTS AND METHODS

Patients

Material from 8 patients was studied: all had typical disease. PB was used in 6 cases, while splenic mononuclear cells were examined in 4 patients (in 2 of these both PB and splenic HCs were tested).

PB was also examined from 6 cases of CLL, 1 case of PLL, 1 HCL variant, and 1 follicular non-Hodgkin's lymphoma (NHL) with peripheral blood involvement. In addition, bone marrow cells from 5 cases of multiple myeloma (MM) were also studied. Normal B lymphocytes were extracted form PB (1 case), and from tonsils which had been surgically removed (4 cases).

Cell preparation

PBM were isolated by density gradient centrifugation over lymphoprep. Splenic and tonsil tissue was dispersed through a wire mesh before centrifugation over lymphoprep (as in Chapter 1). Where T cells formed >5% of HCL PBM they were removed prior to culture using AET-treated SRBC rosetting (as in Chapter 1).

B lymphocytes were purified from tonsil preparations by two rounds of AET-treated SRBC rosetting to remove T lymphocytes; monocytes were then removed by plastic adherence (as in Chapter 1). The resulting populations were >98% CD19 positive B lymphocytes. These B cells were then separated further into their activated (low density) and resting (high density) fractions by density gradient centrifugation over 62.5% percol (as in Chapter 2).

Nucleated bone marrow cells were separated by density gradient sedimentation over 0.1% methyl cellulose.

Cell culture

All cell cultures were performed in complete-RPMI and incubated at 37°C in 5% CO_2 in air.

The choriocarcinoma cell line BeWo was kept in continuous culture and, as the cells were adherent, fed by removing the medium twice a week and replacing with fresh complete-RPMI. On reaching confluence, the cells were subcultured using trypsin.

HL60 cells were also kept in continuous culture in complete-RPMI, and subcultured twice a week.

All B cell cultures were performed at a cell concentration of 2×10^6 cells/ml in complete-RPMI.

Immunophenotyping

Antibodies. MAbs against the following antigens were used; *c-fms* (2E11, a gift from Dr. Lopez), CD11b (Mac-1), CD14 (Leu M3), CD16 (Leu 11a), CD19 (Leu 12), CD20 (Leu 16), CD23 (MHM3, a gift from Prof. McMichael), CD25 (Tac), CD38 (a gift from Dr. Tedder), light chain sIg (κ, λ), DR (a gift from Dr. Smith) and phosphotyrosine (PY20, ICN-Flow).

A polyclonal rabbit antibody to the *c-fms* protein (PEPA 49, Serotec) was also employed.

Surface staining. Cells were stained using indirect immunoflourescence and analysed by FACS, as in Chapter 1.

Internal immunofluorescent staining. Since the 2E11 MAb binds to the cytoplasmic portion of the *c-fms* protein, the cells were permeabilised prior to staining. As the polyclonal antibody (PEPA 49) recognises both internal and external epitopes the cells were also permeabilised prior to staining with this antibody to ensure maximal staining. Cells were permeabilised by incubating for 10 minutes at room temperature with 0.5% paraformaldehyde, followed by a 30 minute incubation with 50% ethanol at 4°C. The cells were then stained using an indirect

immunofluorescent technique employing either FITC-labelled goat anti-mouse, or sheep anti-rabbit, immunoglobulin as a second layer (as in Chapter 1). BeWo and HL60 cells were also stained in this way as positive and negative controls respectively.

APAAP. This technique was used to demonstrate expression of the *c-fms* protein by an alternative methodology, and when the cell numbers were small. Also, the method allowed for morphological identification of the small number of cells expressing the *c-fms* protein which were observed in the non-HC populations.

For all three staining methodologies, the appropriate non-immune IgG1, IgG2a and IgG2b were used as class specific controls.

Immunoprecipitation

C-fms protein. HCs were lysed, and the membrane fraction was immunoprecipitated with the polyclonal antibody to the *c-fms* protein; HL60 and BeWo cells were treated in the same way and used as negative and positive controls respectively. The precipitated proteins were then reduced with β-mercaptoethanol, and SDS-PAGE carried out using a 10% gel. Samples were then Western blotted onto nitrocellulose which was then probed with the MAb to the *c-fms* protein. To visualise this MAb, and hence the *c-fms* protein, the nitrocellulose was treated with ^{125}I-labelled goat anti-mouse immunoglobulin (Bionuclear Services Ltd.). The nitrocellulose was then dried and autoradiographed (Appendix 4.1).

Phosphorylation of the *c-fms* protein. HCs were cultured for 4 hours with and without M-CSF at 1000U/ml. The BeWo cell line, which is known to phosphorylate *c-fms* in response to M-CSF, was used as a positive control (Rettenmier *et al* 1986). To prevent any phosphorylated products being degraded all subsequent procedures were carried out in the presence of phosphatase inhibitors. The M-CSF treated and control cells were each separated into 2 aliquots for further treatment. To look for phosphorylation of the *c-fms* protein, one set of aliquots was lysed and immuno-precipitated with the polyclonal antibody to the *c-fms* protein before being reduced

with β-mercaptoethanol. To examine total protein phosphorylation as a result of M-CSF treatment, the other set of aliquots was reduced with β-mercaptoethanol, without immunoprecipitation. Both sets of samples were then run on SDS-PAGE, using a 10% gel, and Western blotted onto nitrocellulose. The nitrocellulose was probed with the MAb to phosphotyrosine. MAb reactive material was then identified with ^{125}I-labelled goat anti-mouse immunoglobulin and subsequent autoradiography (as above: Appendix 4.2).

Effect of M-CSF and other cytokines on *c-fms* protein expression by HCs

C-fms protein expression was measured after 4, 24 and 48 hours incubation with M-CSF (a gift from Eurocetus), TNFα (a gift from Knoll), or GM-CSF (Ely Lilly) at 5000, 1000 and 100U/ml respectively. M-CSF activity was established by titrating (500 and 5000U/ml) against colony formation at 9 days (Fig. 1); 5637 conditioned medium (10%) was used as a control for colony growth. TNFα was used at the optimum concentration for HC proliferation (as in Chapter 3). GM-CSF was used at a dose which results in the formation of GM colonies (Wong *et al* 1985).

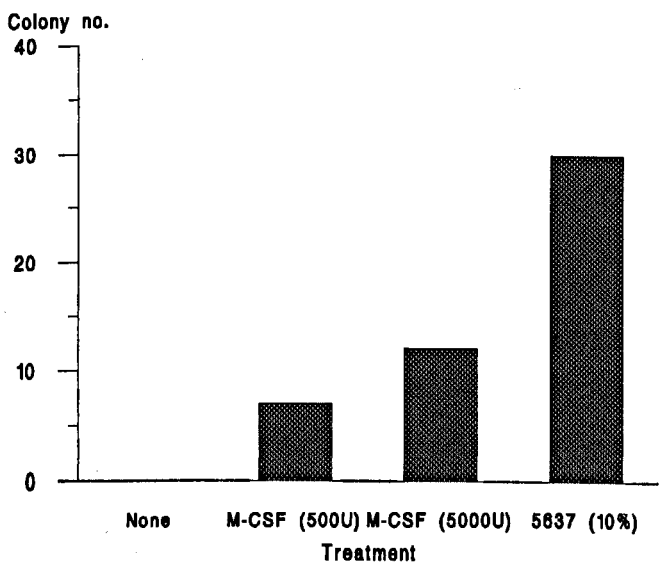


Fig. 1: Effect of M-CSF and 5637 conditioned medium on colony formation by bone marrow mononuclear cells.

Similar experiments were performed with αIFN (100U/ml; as in previous Chapters). *C-fms* protein expression was then measured after 4, 24, 48 and 72 hours.

Functional effects of M-CSF on HCs

Morphology and viability. The morphology of HCs cultured in the presence of M-CSF (5000U/ml) was monitored daily for 20 days, using both *in situ* phase contrast microscopy, and by preparing Romanowsky stained cytocentrifuge slides.

The viability and cell number of HCs cultured with and without M-CSF at 5000U/ml were also measured daily for 20 days using trypan blue dye exclusion.

Immunophenotype. HCs were incubated in the presence or absence of M-CSF (5000U/ml), and stained at 4, 24 and 48 hours using MAbs to the following; *c-fms*, CD11b, CD14, CD16, CD20, CD23, CD25, CD38, DR, κ and λ. MAbs to CD11b, CD14 and CD16 were used as the antigens are either induced, or increased in monocytes in response to M-CSF (Wilman *et al* 1989; Munn *et al* 1990); the other MAbs are to antigens which are altered on either activation, or differentiation, of B lymphocytes.

Proliferation. HCs were incubated with and without M-CSF at 500 and 5000U/ml. In addition, since culture of HC with monocytes results in HC proliferation (Griffiths & Cawley 1991), HCs were incubated in the presence of monocytes (10 monocytes:1 HC) both with, and without, the addition of M-CSF at 1000U/ml. Proliferation was measured by adding 0.5μCi of tritiated thymidine to cultures 18 hours before harvesting at 3, 6 and 9 days onto glass fibre filter papers and scintillation counting (as in Chapter 2).

Immunoglobulin secretion. The supernatants were collected from HCs cultured for 10 days in the presence or absence of M-CSF at 5000U/ml. The supernatants from tonsils stimulated with SAC (0.001%) and IL-2 (100U/ml) in the presence or absence of M-CSF were also collected. Levels of secreted IgG and IgM were then measured in these supernatants using ELISA. Briefly, plates were incubated with antibodies to either IgG or IgM before adding culture supernatants, or standard immunoglobulins in

doubling dilutions. Bound immunoglobulin was visualised using HRP-conjugated goat anti-human immunoglobulin, followed by the substrate o-phenyanaline dichloride. The reaction was stopped with H_2SO_4 and the optical density measured. Calibration lines were drawn using the standard IgG and IgM, and the amounts of immunoglobulins in the culture supernatants were calculated by reference to these lines (Appendix 4.3).

Effects of M-CSF in combination with other cytokines. Since M-CSF alone had little effect on HCs, the effects of co-stimulation with M-CSF and other cytokines were examined. HCs were cultured in the presence or absence of M-CSF at 500 and 5000U/ml together with the following cytokines; IL-2 at 10, 100 and 500U/ml; IL-4 at 100 and 1000U/ml; IL-6 at 1, 50 and 100U/ml; sepharose conjugated anti-μ at 0.1, 1 and 10μg/ml; TNFα at 100 and 1000U/ml; and SAC at 0.001%. TNFα and anti-μ cause maximal HC proliferation at 1000U and 10μg/ml respectively (Griffiths & Cawley 1990). IL-2, IL-4 and IL-6 alone have no direct effect on HCs, therefore the concentrations used were in the range where they exert their effects on normal B cells (Griffiths & Cawley 1990). HC morphology, cell number, viability, phenotype, proliferation, and immunoglobulin secretion were examined in the same way as for direct stimulation with M-CSF.

C-fms protein expression on tonsil cells after stimulation

Since *in vivo* activated tonsil B lymphocytes were found to express the *c-fms* protein, tonsil B cells were activated *in vitro* by incubation with SAC (0.001%) plus IL-2 (100U/ml) or PMA (3.3×10^{-8}M) for 24 hours. To ensure that the B lymphocytes were activated by these mitogens the cells were stained for CD25 and CD23.

As a positive control HL60 cells were cultured for 24 hours in the presence or absence of PMA at 3.3×10^{-8}M, a concentration known to induce *c-fms* expression on these cells (Sariban *et al* 1989).

Functional effects of M-CSF on tonsil B lymphocytes

Tonsil B cells were incubated in the presence or absence of M-CSF (5000U/ml) with or without SAC (0.001%) plus IL-2 (1000U/ml). Proliferation and immunoglobulin secretion were then measured as before.

RESULTS

***C-fms* protein expression in HCL and other lymphoproliferative disorders**

HCs expressed the *c-fms* protein in all cases (8) studied. Between 80 and 90% of HCs were positive by both APAAP (Fig. 2a) and FACS (Fig. 2b) using both the polyclonal and monoclonal antibodies. The intensity of staining (3 - 4 x 10^4 molecules per cell; Table 1a) was comparable to that seen on BeWo cells (5 - 8 x 10^4 molecules per cell).

B cells from other lymphoproliferative disorders did not express the *c-fms* protein (Table 1b). Any positive cells on these APAAPs had the morphological features of monocytes or macrophages and, on their removal by plastic adherence, *c-fms* protein expression was absent (data not shown).

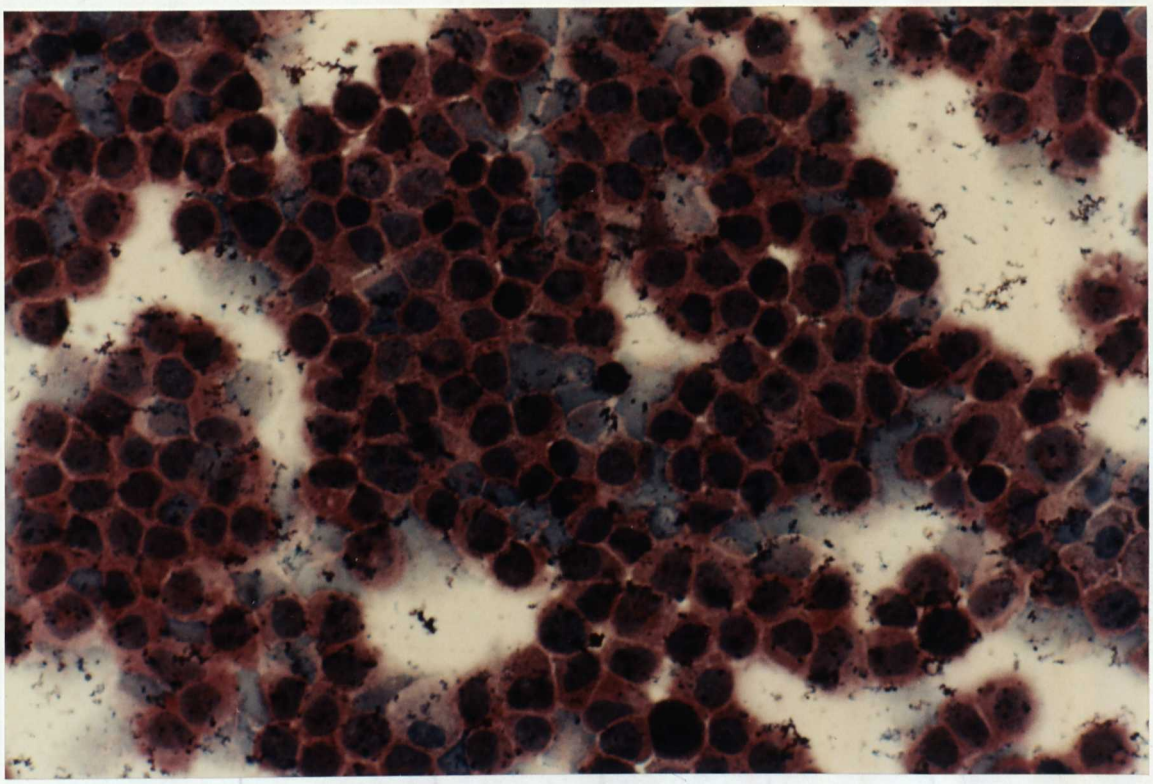
Immunoprecipitation of the *c-fms* protein from HCs and BeWo cells with the 2E11 MAb resulted in the expected 150kD band after SDS-PAGE (Fig. 3); no band was seen with HL60 cells.

***C-fms* protein expression on normal B cells**

C-fms expression was also seen using FACS analysis, on the low density (activated) fraction of tonsil B lymphocytes but not on the high density (resting) fraction (Table 2). Normal PB B cells did not express the *c-fms* protein (Table 2).

Stimulation of tonsil B cells with both PMA and SAC plus IL-2 caused an increase in the levels of the *c-fms* protein (Fig. 4). Only a proportion of the activated cells expressed the *c-fms* protein since a larger percentage of cells were positive for the CD23 and CD25 activation antigens (Fig. 4).

a. APAAP



b. FACS

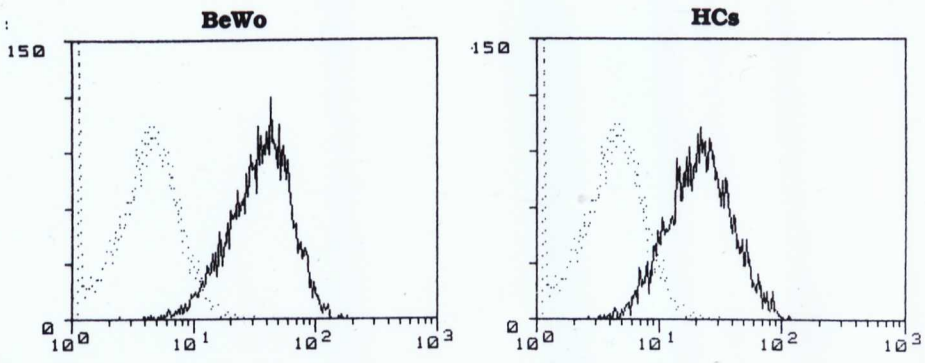


Fig. 2: Expression of the *c-fms* protein by HCs. (.....IgG1 control, ____ *c-fms*)

a. Cell types positive for the *c-fms* protein

HC no.	APAAP[a] (%)	FACS[a]	
		%	Mean x 10^4
HC 1	62	90	4.3
HC 3	94	ND	
HC 7	96	ND	
HC 8	96	ND	
HC 10	94	ND	
HC 10s	81	ND	
HC 12	77	51	3.0
HC 12s	ND	77	3.6
HC 13	61	80	4.0
HC 14	16	80	3.0

b. Cell types negative for the *c-fms* protein

Cell type	*c-fms*[b]	CD14	CD19
MM 1	3	6	ND
MM 2	7	5	ND
MM 3	4	9	ND
MM 4	11	14	ND
MM 5	6	7	ND
CLL 1	3	3	94
CLL 2	7	7	28
CLL 3	3	3	87
CLL 4	5	5	95
CLL 5	3	4	95
CLL 6	3	9	68
HCL like	10	15	20
CLL like	0	0	89
PLL	4	5	90
NHL	8	9	ND

Table 1: *C-fms* protein expression on B cells from HCL and other lymphoproliferative disorders. a) Purified HCs were used for FACS analysis, whereas PB was used for APAAP. b) All *c-fms* positive cells on APAAP were monocytes/macrophages. ND=not done.

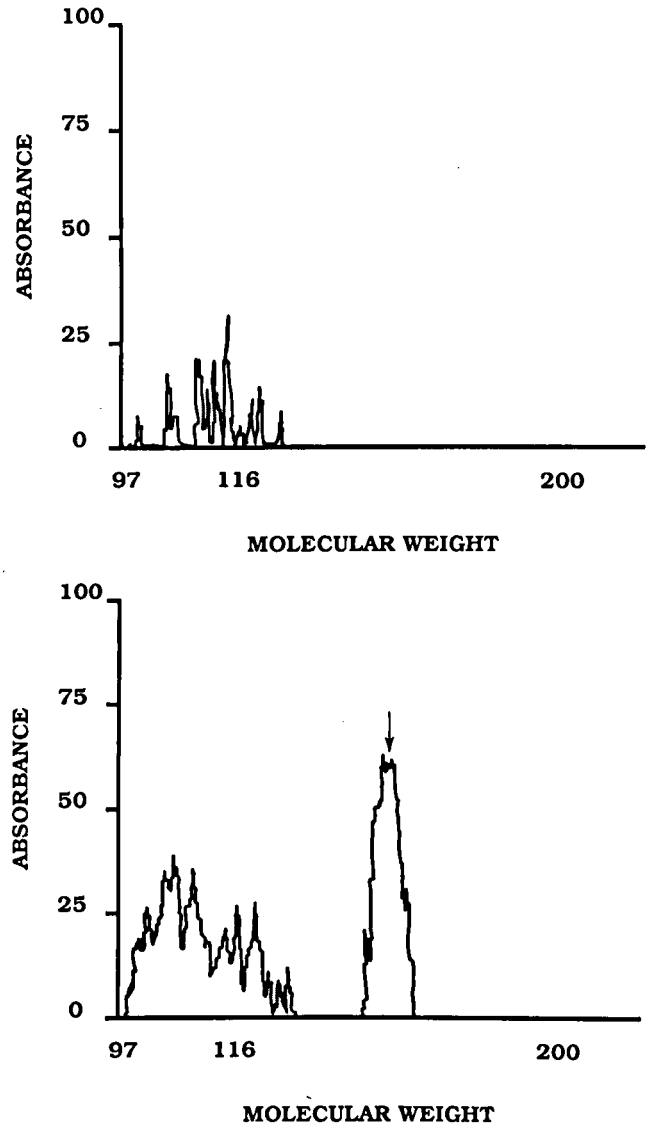


Fig. 3: Densitometry profile of the *c-fms* protein immunoprecipitated from HCs.(Top IgG1 control; bottom = *c-fms* ↓)

Cell type	Density	CD23		CD25		*c-fms*	
		%	Mean x 10^4	%	Mean x 10^4	%	Mean x 10^4
Tonsil 1	low	55	4.2	51	6.2	70	5
	high	0		0		20	<2.5[a]
Tonsil 2	low	60	3.5	62	5.3	67	8
	high	0		0		20	<2.5[a]
PB[b]		0		0		1	

Table 2: Effect of activation on *c-fms* protein expression by normal B cells. a) 2.5 x 10^4 is the limit of detection of the FACS. b) PB was not separated by density.

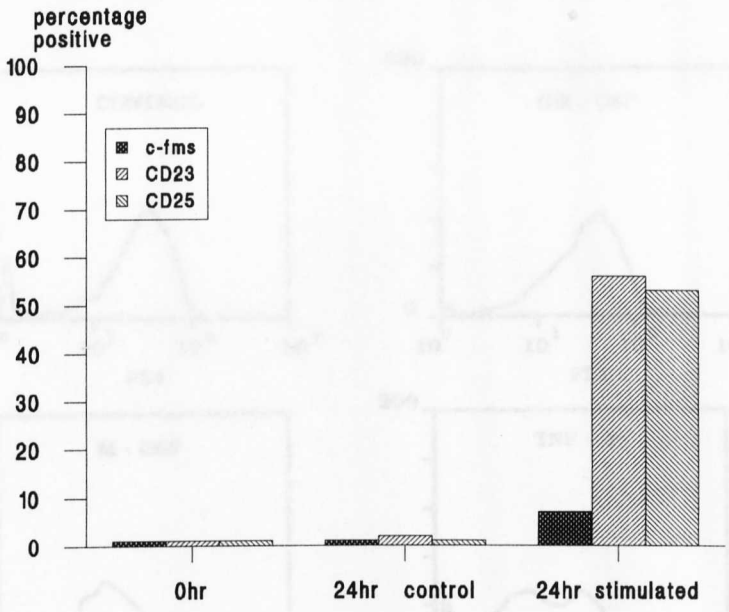


Fig. 4: Effect of stimulation with SAC plus IL-2 on *c-fms* protein expression by tonsil B cells.

HL60 cells did not express the *c-fms* protein; however, stimulation with PMA induced expression with approximately 50% of cells becoming positive (12 x 10^4 molecules/cell).

Effect of M-CSF and other cytokines on *c-fms* protein expression by HCs

Culture of HCs with M-CSF caused down regulation of its receptor, the *c-fms* protein, at 4 hours (Fig. 5). From 24 hours onwards *c-fms* protein expression in untreated cultures had decreased, and M-CSF treatment had no effect on this decline (data not shown). To assess the specificity of the down regulation of *c-fms* protein expression by HCs in response to M-CSF, HCs were cultured with both TNFα and GM-CSF. TNFα caused a decrease in *c-fms* protein expression similar to that produced by M-CSF (Fig. 5). However, GM-CSF had no effect (Fig. 5).

αIFN had no consistent effect on the expression of the *c-fms* protein by HCs during the 3 days of incubation (Table 3). As before, culture of HCs in the absence of cytokines was associated with a decline in *c-fms* protein expression; this was unaffected by αIFN (Table 3).

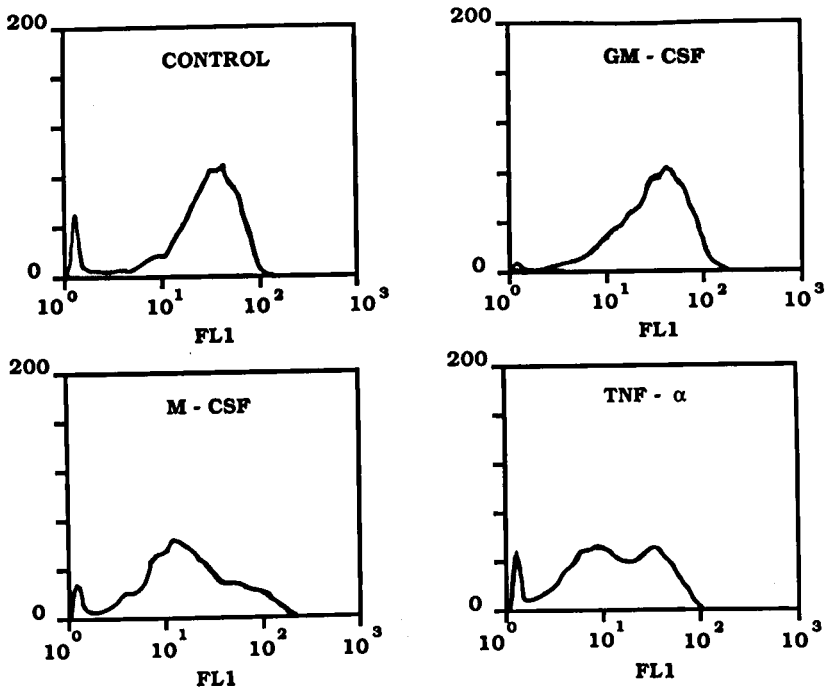


Fig. 5: Effect of cytokines on *c-fms* protein expression by HCs.

HC no.	αIFN	4 hour		24 hour[a]	
		%	Mean x 10^4	%	Mean x 10^4
1	-	35	<2.5	36	<2.5
	+	33	<2.5	37	<2.5
12	-	90	32	20	4
	+	90	27	17	4
12s	-	77	26	23	3
	+	80	28	34	4
13	-	11	<2.5	7	<2.5
	+	18	<2.5	8	<2.5

Table 3: Effect of αIFN on *c-fms* protein expression by HCs. a) Levels remained similar up to 72 hours.

Incubation of HCs with IL-2, IL-4, IL-6, SAC or anti-μ had no effect on *c-fms* protein expression (Table 4). None of these factors affected the M-CSF induced decrease in *c-fms* protein expression (Table 4).

Cytokine	M-CSF	%[a]	Mean x 10^4[a]
none	-	51±5	6±8
	+	29±8	3±1
IL-2	-	48±36	9±4
	+	10±3	<2.5
IL-4	-	38±43	5±3
	+	16±6	<2.5
IL-6	-	47±4	8±5
	+	24±14	<2.5
SAC	-	44±39	12±8
	+	35±45	<2.5
Anti-μ	-	35±45	6±4
	+	22±3	<2.5

Table 4: Effect of IL-2, IL-4, IL-6, SAC and anti-μ ± M-CSF (5000U/ml) on *c-fms* protein expression by HCs. a) Values = mean of 3 experiments.

Functional effects of M-CSF on HCs

Phosphorylation of the *c-fms* protein. The *c-fms* protein was not phosphorylated on untreated HCs; however, treatment with M-CSF caused phosphorylation of a number of proteins, including the 150kD *c-fms* protein (Fig. 6). A similar pattern of phosphorylation was observed in the control BeWo cells (Fig. 6).

Morphology/viability. After 9 days the number of viable cells rapidly declined, whether or not M-CSF was present. As the viability decreased, both the number and size of the HC clumps and the number of adherent HCs decreased; M-CSF had no effect on these changes (data not shown).

Culture of cells with TNFα, SAC or anti-μ caused an increase in the number of HC clumps; TNFα also caused an increase in HC adhesion. None of the other

cytokines had any morphological effect on HCs. Addition of M-CSF again had no effect (data not shown).

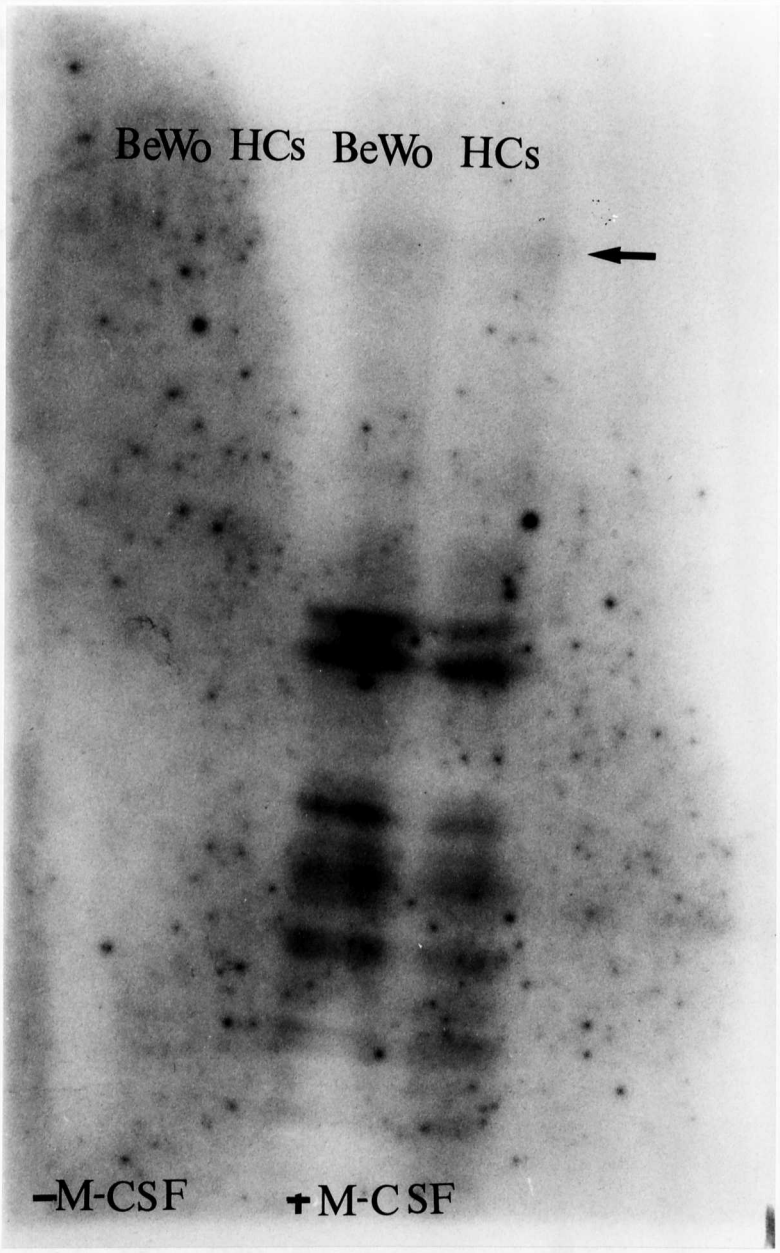


Fig. 6: Phosphorylation of the *c-fms* protein in response to M-CSF on HCs and BeWo cells.

Culture of HCs (3 cases) with either IL-2, IL-4, IL-6, SAC, anti-μ or TNFα had no effect on the viability of the HCs (data not shown). Viability again rapidly declined after 9 days and M-CSF had no effect on this decline (data not shown).

Immunophenotype. Apart from the previously mentioned decrease in *c-fms* protein expression, M-CSF had no effect on the phenotype of HCs at any of the time points tested (Table 5). IL-6, SAC, TNFα and anti-μ all caused reduced CD25 expression: no consistent changes were seen with any of the other cytokines, and co-culture with M-CSF had no additional effect (Table 6).

Antigen	M-CSF	%[a]	Mean x 10^4[a]
CD11b	-	12±8	<2.5
	+	18±7	4±1
CD14	-	1±1	<2.5
	+	0±1	<2.5
CD16	-	22±15	6±5
	+	22±11	6±2
CD20	-	91±5	26±11
	+	89±4	<2.5
CD23	-	6±4	<2.5
	+	7±1	<2.5
CD25	-	55±36	7±3
	+	73±27	8±1
CD38	-	6±4	<2.5
	+	4±4	<2.5
κ	-	95±1	17±3
	+	95±2	19±4
DR	-	85±4	50±20
	+	87±1	50±10

Table 5: Effect of M-CSF on HC phenotype at 24 hours. (Values were comparable at 4 and 48 hours.) a) Values are mean ± SEM for 2 patients.

Antigen	M-CSF	IL-2[a]	IL-4[a]	IL-6[a]	Anti-μ[a]	TNFα[a]	SAC[a]
CD11b	-	8±3	4±1	5±2	4±1	4±1	6±4
	+	6±2	5±1	7±2	5±1	4±2	6±2
CD14	-	0	0	0	0	0	0
	+	0	0	0	0	0	0
CD16	-	0	0	0	0	0	4±2
	+	0	0	5±2	0	0	0
CD20	-	16±3	15±1	240±1	24±2	18±3	14±1
	+	19±6	16±1	23±3	13±5	12±4	16±5
CD23	-	4±2	4±1	5±2	0	0	0
	+	0	0	0	0	0	0
CD25	-	4±3	4±1	6±3	7±2	0	4±2
	+	7±1	6±1	5±2	6±1	5±3	3±2
CD38	-	0	0	0	0	0	0
	+	0	0	0	0	0	0
DR	-	32±6	34±10	31±2	24±5	32±14	18±4
	+	32±2	42±1	30±0	26±6	32±12	26±3

Table 6: Effect of B cell mitogens ± M-CSF on HC antigen expression. a) Mean fluorescence x 10^4 ± SEM of 3 patients.

Proliferation. No significant proliferation was seen in HCs cultured either with or without the addition of M-CSF for up to 9 days (Table 7).

HC no.	M-CSF[a]	3 day[b]	6 day[b]
12	-	735±48	539±57
	+	738±18	655±35
12s	-	502±17	875±53
	+	458±38	523±93
13	-	172±13	105±8
	+	156±4	95±36

Table 7: Effect of M-CSF on HC proliferation. a) M-CSF at 5000U/ml. b) CPM ±SEM.

SAC, anti-μ and TNFα caused HC proliferation in the majority of cases and addition of M-CSF had no effect on this proliferation (Table 8a). IL-2, IL-4, and IL-6, in general had no effect on HC proliferation (Table 8b); M-CSF did not synergise with any of these factors to cause HC proliferation (Table 8b).

a. Mitogens that induce HC proliferation

HC no.	M-CSF[b]	3 day SAC[a]	3 day Anti-μ[a]	3 day TNFα[a]	6 day SAC	6 day Anti-μ	6 day TNFα
1	-	1940±162	342±40	182±3	1512±65	1590±297	2099±193
	+	2019±348	307±392	144±13	1451±288	2355±563	2236±57
12	-	954±150	161±50	168±21	1899±145	706±99	3271±282
	+	743±56	142±27	171±20	1669±32	633±145	3106±252
12s	-	4231±89	3038±715	540±52	723±1	816±34	1094±312
	+	3976±238	2561±223	598±42	654±27	805±15	2230±66

b. Mitogens that do not cause HC proliferation

HC no.	M-CSF[b]	3 day IL-2[b]	3 day IL-4[b]	3 day IL-6[b]	9 day IL-2	9 day IL-4	9 day IL-6
1	-	289±59	114±11	ND	969±190	607±100	ND
	+	384±30	151±1	ND	1508±367	659±140	ND
12	-	125±20	162±41	108±21	1425±527	508±149	112±7
	+	140±16	129±6	127±15	1326±188	625±40	143±5
12s	-	746±146	480±24	402±45	3499±919	613±32	299±50
	+	659±52	485±41	344±5	2839±252	532±59	348±32

Table 8: Effect of M-CSF ± B cell mitogens on HC proliferation. a) SAC 0.001%; anti-μ 10μg/ml; TNFα 1000U/ml. Peak proliferative response was seen at these doses. b) M-CSF 5000U/ml; IL-2 500U/ml; IL-6 50U/ml. No differences were seen with treatment at other doses.

Addition of monocytes to HCs caused proliferation at 9 days (Table 9); M-CSF had no effect on this monocyte induced HC proliferation (Table 9).

HC no.	Mono-cytes	M-CSF	3 day[a]	9 day[a]
1	-	-	480±74	156±49
		+	454±11	141±13
	+	-	413±30	2213±0
		+	388±47	3312±282
12	-	-	633±144	1711±200
		+	816±115	2363±800
	+	-	2266±329	7184±390
		+	2397±501	8224±1334
12s	-	-	183±32	222±108
		+	226±39	253±4
	+	-	738±134	3121±650
		+	883±202	3549±1257

Table 9: Effect of M-CSF on monocyte induced HC proliferation. a) CPM ± SEM.

Immunoglobulin secretion. After 10 days of culture HCs produced only background levels of immunoglobulin secretion (Fig. 7). Addition of M-CSF to HCs did not induce immunoglobulin secretion (Fig. 7).

In addition, incubation of HCs for 10 days with either IL-2, IL-4, IL-6, SAC anti-μ, or TNFα did not induce immunoglobulin secretion by HCs (Table 10); addition of M-CSF had no effect (Table 10).

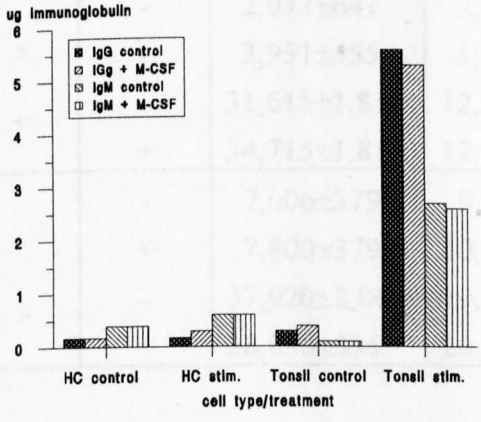


Fig. 7: Effect of stimulation ± M-CSF on HC and tonsil immunoglobulin secretion. stim.=stimulated.

Mitogen	M-CSF	IgG (ng/ml)	IgM (ng/ml)
IL-2	-	142±98	44±9
	+	120±100	63±28
IL-4	-	120±20	43±23
	+	118±83	60±40
IL-6	-	210±100	20±10
	+	230±130	10±5
Anti-μ	-	145±85	25±15
	+	165±104	19±9
TNFα	-	110±72	105±45
	+	143±33	129±71
SAC	-	240±136	272±229
	+	227±165	286±215

Table 10: Effect of mitogens ± M-CSF on HC immunoglobulin secretion

Effect of M-CSF on tonsil B lymphocyte proliferation and differentiation

Incubation of tonsil B lymphocytes with M-CSF alone did not induce proliferation (Table 11). M-CSF had no effect on the proliferation (Table 11) or immunoglobulin secretion induced by SAC plus IL-2 (Fig. 7).

Tonsil no.	SAC + IL-2	M-CSF	3 day	9 day
1	-	-	2,073±641	2,294±84
		+	3,931±455	1,959±226
	+	-	31,615±1,812	12,525±1,449
		+	34,715±1,812	12,196±71
2	-	-	7,006±379	9,838±1,082
		+	7,800±379	10,498±1,120
	+	-	37,920±2,060	19,581±1,879
		+	38,950±335	20,512±1,882

Table 11: Effect of M-CSF on SAC plus IL-2 induced B lymphocyte proliferation.

DISCUSSION

The results of this Chapter clearly demonstrate that HCs posses the M-CSF receptor, the *c-fms* protein. Expression of the *c-fms* protein by HCs was demonstrated using two antibodies, and staining by both immunofluorescence and APAAP. The *c-fms* protein on HCs had a molecular weight of 150kD, which corresponds to its molecular weight on other cell types (Sherr *et al* 1990). Expression of the *c-fms* protein was specific to HCs, as all the other mature B cell lymphoproliferative disorders examined (multiple myeloma, PLL, CLL CLL-like and HCL-like) lacked this proto-oncogene.

Since HCs are activated B cells, resting and activated normal B cells were examined for expression of the *c-fms* protein. *In vivo* activated tonsil B cells expressed the *c-fms* protein, whereas the corresponding resting cells did not. Activation of tonsil B lymphocytes *in vitro* resulted in enhanced expression of the *c-fms* protein, however the proportion of positive cells was less than in the *in vivo* activated tonsil B cells. The reasons for this difference are unclear; it may be that the tissue environment of tonsil B lymphocytes plays a part in their antigen expression.

Among haemopoietic cells, expression of *c-fms* was thought to be confined to cells of the monocyte/macrophage lineage (Ashmun *et al* 1989; Sariban *et al* 1985). Recently however, *c-fms* expression has been observed on a Hodgkin's lymphoma derived cell line (Paietta *et al* 1990). This observation, together with those in this Chapter, suggest that the *c-fms* protein may be more generally expressed among activated cells of the haemotopoietic system.

Binding of M-CSF to the *c-fms* protein on HCs caused reduced receptor expression; this reduced expression in response to ligand binding is also seen on cells of the monocyte/macrophage lineage (Sherr *et al* 1988; Rettenmier 1987), and is due to receptor internalisation and degradation (Tamura *et al* 1987). Expression of the *c-fms* protein on HCs, as in monocytes/macrophages (Sherman *et al* 1990; Sariban *et al* 1989), was down regulated by TNFα, but not by GM-CSF. However, the cytokine αIFN had no effect on *c-fms* protein expression. In the presence and absence of

cytokines, expression of the *c-fms* protein progressively declined between 4 and 48 hours in culture. Since the M-CSF receptor is highly labile, and requires an active metabolism to maintain its expression (Sherr 1990), this decline is probably due to the fact that HCs are metabolically inactive after 18 hours in culture.

Binding of M-CSF to the *c-fms* protein on HCs caused rapid phosphorylation of both the receptor and a number of other proteins on tyrosine. This phosphorylation is also seen on monocytes (Yeung *et al* 1987; Downing *et al* 1988), and is due to the tyrosine kinase domain on the *c-fms* protein (Tamura *et al* 1989). Both the phosphorylation and down regulation of the *c-fms* protein in response to M-CSF indicate that the cytokine stimulates HCs; therefore the consequences of this stimulation were sought.

Both monocytes and macrophages proliferate in response to M-CSF (Sherr *et al* 1989). Levels of M-CSF which are too low to cause proliferation maintain the viability of both monocytes and macrophages (Sherr 1989; Tushinski & Stanley 1985), and increase their long term survival (Yeung *et al* 1990; Williams *et al* 1990). Administration of M-CSF also alters antigen expression in monocytes, where it causes decreased levels of class II MHC (Willman *et al* 1989). When M-CSF is administered to myeloid progenitor cells transfected with *c-fms* it causes differentiation into monocytes, but not macrophages (Pierce *et al* 1990); this differentiation is accompanied by changes in morphology, and increased expression of the surface antigens CD11b, CD14 and CD16 (Munn *et al* 1990). If M-CSF is removed from cultures containing these transfected cells they revert to their immature state (Peirce *et al* 1990). Treatment of HCs with M-CSF (alone, or in combination with IL-2, IL-4, IL-6, SAC, anti-μ, TNFα or monocytes) did not induce proliferation, differentiation (as indicated by immunoglobulin secretion and antigen expression), increased survival, or changes in either morphology or any of the surface antigens altered by M-CSF in monocytes. M-CSF also had no effect on the proliferation or immunoglobulin secretion of *in vitro* activated tonsil B lymphocytes.

In conclusion, the present studies indicate that both HCs and activated normal B lymphocytes express the *c-fms* protein, and that its ligand M-CSF stimulates HCs. Although the functional studies of M-CSF reported here failed to indicate a possible role for the cytokine, work performed in this Department since the completion of this thesis indicated that M-CSF is involved in the adhesion and motility of HCs (Burthem *et al* 1993).

GENERAL CONCLUSION

The work presented in this thesis showed that αIFN has a direct effect on HCs. The cytokine induced profound changes in the phenotype of HCs. The pattern of these changes was difficult to interpret. However, it may be that the cytokine induces the partial activation/differentiation of HCs. Furthermore, the significance of these changes in relationship to the mechanisms by which αIFN exerts its effects in HCL remains unclear.

In order to ascertain whether αIFN was inducing HC activation or differentiation the levels of the protein products of the proto-oncogenes *c-myc* and *c-fos* were investigated. αIFN did not affect the levels of these proteins in unstimulated HCs; however, the cytokine caused reduced expression of both the *c-myc* and *c-fos* proteins in HCs which had been stimulated to proliferate. This reduction in the expression of the *c-myc* and *c-fos* proteins by HCs was accompanied by growth arrest. Since these onco-proteins are a vital component of the signalling pathway for cell division these results indicate that αIFN exerts its anti-proliferative effects in HCL by a pathway involving reduced expression of these two proto-oncogenes.

The fact that αIFN inhibits HC proliferation prompted a search for the *in vivo* stimulus for HC growth. It is well known that HCs are associated *in vivo* with accessory cells, in particular endothelial cells and macrophages. The work of this thesis attaches pathophysiological significance to this association, indicating that these accessory cells are responsible for inducing HC proliferation. This proliferation is mediated by a combination of the cytokine TNFα and by binding of HCs to endothelial cells via the leukocyte integrins CD11b/CD18 and CD11c/CD18 binding to CD54 and the CD11c ligand respectively. The binding via CD11c may indicate why HCs express a large amount of this protein, and provides a system to investigate what the ligand for this adhesion molecule might be. Work is currently underway on this.

The finding that HCs and normal activated B cells express the *c-fms* protein, the M-CSF receptor, was the first report of this oncogene being found on haemopoietic cells other than those of the myeloid lineage. Although the M-CSF receptor on HCs

was shown to be active, the cytokine did not induce HC proliferation, differentiation, increased survival, or changes in morphology or phenotype. These results suggested that M-CSF has another function on B cells; work performed by a colleague in the Department indicates that M-CSF is involved in the migration of HCs.

APPENDIX

2: ONCOGENE EXPRESSION

2.1: Isolation of nuclei and staining for oncoprotein expression

Buffers and solutions

Stock citrate - 3.4mM trisodium citrate (BDH)
0.1% Nonidet P40 (BDH)
1.5mM Sperminetetrahydrochloride (STC; Sigma)
pH 7.6

Solution A - 0.3mg/ml trypsin (Sigma)
In stock solution

Solution B - 0.1mg/ml Ribonuclease A (Sigma)
0.5mg/ml Trypsin inhibitor (Sigma)

Solution C - 1.628mg/ml STC
In stock solution

Propidium iodide (PI) - 0.42mg/ml PI (Sigma)
In solution C

Method

1. Cells were fixed in methanol for between 18 hours and 7 days.
2. Cells were pelleted and the liquid removed.
3. Cells were incubated for 15 minutes at room temperature with 2mls of solution A.
4. 2mls of solution B was added and cells were incubated for 15 minutes at room temperature.
5. 4mls of solution C was added and cells were pelleted, 15 minutes at 1000g at 4°C and the supernatant removed.

6. MAbs were diluted in stock citrate solution (10μg/ml *c-myc* and 5μg/ml *c-fos)* and 100μl added to nuclei which were then incubated on ice for 1 hour.

7. Nuclei were washed twice in 10mls of solution C and the supernatant removed.

8. 50μl of 10% FITC conjugated to either goat anti-mouse, or rabbit anti-sheep, immunoglobulin was added and the nuclei incubated on ice for 30 minutes.

9. Nuclei were washed twice in solution C and the supernatant removed.

10. 1ml of PI solution was added and nuclei were left on ice for at least 5 minutes prior to analysis by FACS.

3: TNFα AND ACCESSORY CELLS

3.1 APAAP and HRP double staining

Buffers and solutions.

Tris HCl - 0.025M
pH 7.6

10x Tris - 0.5M
pH 7.6

NaCl - 0.15M

TBS - 10x Tris diluted 1:10 with 0.15M NaCl

Tris HCl - 0.1M Tris
pH 8.2

DAB - 12.5mg in 25mls Tris HCl
pH 8.2
20μl H_2O_2

APAAP substrate - a) 2mg/ml napthol AS-MX phosphate

+ 200µl N'N' dimethlyformamide

b) 10mg fast red TR salt

+ 10mls Tris HCl pH 7.6

Mix a + b + 10µl 1M levamisole

Staining

All antibodies had been titrated to saturating levels.

1. Cytospins were fixed for 3 minutes in ice cold acetone and rinsed in TBS.
2. 30µl of Ki67 was added and the slides were incubated for 30 minutes at room temperature and then rinsed in TBS.
3. 30µl of HRP conjugated rabbit anti-mouse Ig was added, slides were incubated at room temperature for 30 minutes and then rinsed in TBS.
4. DAB substrate was filtered onto slides and incubated at room temperature for 10-15 minutes and then washed in TBS.
5. 20µl of MAb was added, slides were incubated at room temperature for 30 minutes and washed in TBS.
6. 40µl of rabbit anti-mouse Ig containing 10% human AB serum was added, slides were incubated at room temperature for 30 minutes and rinsed in TBS.
7. 40µl of APAAP complex was added, slides were incubated at room temperature for 30 minutes and rinsed in TBS.
8. Steps 6 & 7 were repeated with 10 minute incubations.
9. APAAP substrate was filtered onto slides which were then incubated for 20 minutes at room temperature and rinsed in TBS.
10. Cells were counterstained with Haematoxylin for 1 minute and the colour developed in tap water.

3.2 Irradiation doses used to kill accessory cells

a. FV1

Dose (rads)	CPM ± SEM
0	28,826±2039
3200	133±7
3600	109±5
4000	110±26

b. INT 409

Dose (rads)	CPM ± SEM
0	167,797±5262
4000	83,620±1330
8000	94,499±3956
40,000	368±13

C-FMS

4.1: Immunoprecipitation of *c-fms*

Buffers and solutions

Lysis buffer - 150mM NaCl

10mM Tris pH 8.1

1mM EDTA

0.5% Nonidet (NP) p40

1mM PMSF

Wash buffer - 0.5mM LiCl

100mM Tris pH 8

0.5% NP 40

DSSB - 20% v/v glycerol

10% v/w sodium dodecyl sulphate (SDS)

25% stacking gel buffer

0.1mM EDTA

4.5g bromophenol blue

Running gel buffer - 1.5mM Tris

4% SDS

Running gel - acrylamide at required %

25% running gel buffer

0.1% TMED

0.3% ammonium persulphate (APS)

in H_2O_2

Stacking gel buffer - 0.5M Tris

4% SDS

Stacking gel - 2% acrylamide

25% stacking gel buffer

0.1% TMED

0.3% APS

in H_2O_2

Electrode buffer - 1% SDS

0.2M glycine

25mM Tris

pH 8.3

Fixative - 10% trichloroacetic acid

10% acetic acid

in H_2O_2

Coomasie blue stain - 0.12% w/v brilliant blue

25% ethanol

10% acetic acid

Destain - 10% methanol

10% acetic acid

in H_2O_2

Immunoprecipitation

1. $1x10^6$ cells were incubated in 1ml of lysis buffer at 4°C for 30 minutes.
2. Cell debris was removed by centrifugation at 16,000g for 30 minutes at 4°C.
3. HC immunoglobulin was removed by incubating lysate with pansorbin for 1 hour at 4°C.
4. Immunoglobulin and pansorbin were removed by pelleting them at 16,000g for 30 minutes at 4°C.
5. Lysate was then incubated overnight with MAbs at 10µg/ml at 4°C.
6. Immunoprecipitated proteins were absorbed onto protein G Sepharose (PGS) by incubating at 4°C for 1 hour.
7. Immunoprecipitated proteins on PGS were washed 4 times in wash buffer to remove non-bound proteins.

Labelling

1. Iodogen was added to the Immunoprecipitated proteins at 0.05mg/ml.
2. 25μg of ^{125}I was added together with 20μl/ml of 0.12% H_2O_2 and incubated at room temperature for 5 minutes.
3. A further 20μl/ml of H_2O_2 was added and the solutions were incubated for a further 5 minutes.
4. Immunoprecipitates were washed 5 times in wash buffer.
5. Immunoprecipitates were resuspended in DSSB and 10% β-mercaptoethanol and boiled for 5 minutes.

SDS-PAGE

1. A 5 to 20% gradient polyacrylamide gel was cast.
2. Gel was run overnight with SDS as electrode buffer.
3. Gel was fixed for 5 minutes before being stained for 5 minutes in Coomasi blue.
4. The gel was destained, then dried and autoradiographed.

4.2 Western blotting

Buffers and solutions

Phosphatase inhibitors - 10mM NaFl
10mM $Na_2P_3O_7.10H_2O$
0.4M Na_2VO_4
0.4M EDTA

Transfer buffer - 25mM Tris
192mM glycine
0.1% SDS
20% methanol
pH 8 - 8.5

TBS - 10mM Tris

500mM NaCl

pH 8

Tween-TBS - 0.5% Tween 20

in TBS

Sample preparation.

1. Cells for total protein (±M-CSF) were pelleted and boiled for 5 minutes in DSSB plus phosphatase inhibitors.
2. *C-fms* was immunoprecipitated from the other samples (±M-CSF) as before with the addition of phosphatase inhibitors to the lysis buffer.
3. Precipitates from 2 were boiled in DSSB plus 10% β-mercaptoethanol and frozen at -20°C prior to use.

SDS-PAGE

1. SDS-PAGE was performed as before using a 10% gel.

Western blotting

1. Spacer gel and excess gel on either side of the sample lanes and below the leading front was cut off and discarded.
2. Gel was incubated in transfer buffer, shaking, at room temperature for 15 minutes.
3. Filter paper and nitrocellulose were cut to the size of the gel.
4. A sandwhich was made using the folowing layers, filter paper, nitrocellulose, the gel and then more filter paper; all were soaked in transfer buffer.
5. Electrodes were wet with water and the nitrocellulose sandwich from 4 was placed on the electrodes with the nitrocellulose nearest the anode.
6. The blot was run overnight at 4°C.
7. The nitrocellulose was washed twice in TBS.
8. The nitrocellulose was blocked with 5% BSA in TBS at room temperature for 1 hour.

Iodination of goat anti-mouse immunoglobulin.

1. Sephadex G25 column was washed.
2. Iodobead was washed twice with phosphate buffer (50mM Na_2HPO_4) and dried on filter paper.
3. 4mBq of ^{125}I was incubated with the iodobead for 5 minutes at room temperature.
4. 200μg of goat anti-mouse immunoglobulin was then added and incubated at room temperature for 15 minutes.
5. The solution from 5 was passed through the G25 column and collected in 0.5ml aliquots.
6. Aliquots were counted and fractions 6-8 were found to contain the iodinated protein. These fractions were pooled and frozen at -20°C prior to use.

Probing

1. The nitrocellulose was incubated overnight with the anti-phosphotyrosine MAb at 1μg/ml in Tween(1%)-TBS containing 1% BSA.
2. Nitrocellulose was washed twice in TBS.
3. Nitrocellulose was incubated for 2 hours at room temperature with the iodinated goat anti-mouse immunoglobulin at 20μg/ ml in T-TBS containing 1% BSA.
4. Nitrocellulose was washed 10 times in T-TBS and twice in TBS.
5. Nitrocellulose was dried and autoradiographed.

4.3 ELISA

Buffers and solutions

Borate saline (BS) - 61.8g boric acid
95.4g borax
43.8g NaCl
in 10l H_2O

BS/BSA - 5g BSA
in 1l BS

Citrate buffer - 10.08g citric acid

17.8g $Na_2HPO_4.10H_2O$

980mls H_2O

pH 5

Substrate - 5µl H_2O

400µl o-phenlydiamine dichloride (OPD)

25 mls citrate buffer

Method

1. 96 well ELISA plates were coated overnight with either anti-human IgG (3.5mg/ml) or IgM (5mg/ml) at 4°C.
2. Antibody was removed and plates were blocked with BS/BSA for 1 hour at room temperature.
3. Plates were washed in tap water and excess blotted off.
4. 100µl BS/BSA was added to each well.
5. Standard IgG and IgM at 20 and 11.4ng/ml respectively were added to 2 wells on the top row.
6. Culture supernatants were added in triplicate to wells on the top row and doubling dilutions were made down the plate.
7. Plates were incubated for 3 hours at 37°C and washed in tap water as before.
8. HRP-conjugated goat anti-human immunoglobulin at 5ng/ml was added to wells and incubated for 1 hour at 37°C.
9. Plates were washed, OPD substrate was added to the wells, and plates were incubated for 30 minutes at room temperature.
10. The reaction was stopped by adding an equal amount of 0.12% H_2SO_4.
11. Plates were read at 492 nm and the amount of immunoglobulin calculated using a calibration line drawn using the standard immunoglobulins.

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