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Expression of BCR–ABL in M1 myeloid leukemia cells induces differentiation without arresting proliferation

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The mechanism leading to the expanding population of maturing myeloid cells which characterises chronic myeloid leukemia (CML) remains obscure. Because of its ability to mimic the proliferative and cell survival functions of hematopoietic growth factors, we hypothesized that the oncogene activated in CML, BCR–ABL, might also influence differentiation. To test this hypothesis, we examined the effects of expressing BCR–ABL on the myeloid differentiation of murine M1 leukemic cells, which cease dividing and differentiate into macrophages in the presence of the cytokines leukemia inhibitory factor (LIF) or interleukin (IL)-6. We found that BCR–ABL induced macrophage differentiation in M1 cells, accompanied by increased expression of macrophage cell surface markers and the acquisition of phagocytic ability. Interestingly, clones of M1 cells which expressed BCR–ABL remained in cell cycle and were refractory to the growth inhibition and apoptosis induced by IL-6 or LIF in parental M1 cells. These cells also expressed inappropriately high levels of c-MYC mRNA for their degree of differentiation, which may have been important in maintaining cellular proliferation. These data suggest that BCR–ABL can stimulate both differentiation and proliferation and that these characterisitics may contribute to the phenotype observed in CML.

Keywords: BCR–ABL oncogene; M1 cell differentiation

Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative syndrome originating from the neoplastic transformation of a multipotential progenitor cell. Clinically, there is an initial chronic phase marked by the progressive accumulation of granulocytes and their precursors in the blood and hematopoietic organs. Several years later, following a brief period of disease acceleration, there is transformation into an acute myeloid or lymphoid leukemia. The t(9;22) translocation, which is the cytogenetic hallmark of CML, creates a fusion gene, *BCR–ABL*, which encodes a 210 kD protein with enhanced tyrosine kinase activity compared to endogenous c-ABL (Groffen and Heisterkamp, 1987).

During the last few years, evidence has emerged indicating that BCR–ABL interacts with a large number of molecules in signal transduction pathways downstream of growth factor receptors (see Tauchi and Broxmeyer, 1995 for a review), probably substituting for activated receptor- or non-receptor-tyrosine kinases. For example, BCR–ABL directly or indirectly activates the RAS/mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K) and Janus kinase (JAK)/signal transducers and activators of transcription (STAT) pathways, stimulates expression of c-MYC and D-type cyclins and phosphorylates focal adhesion proteins (reviewed in Tauchi and Broxmeyer, 1995; Elefanty *et al*., 1997). The activation of these signalling cascades by BCR–ABL abrogates cytokine dependence in factor-dependent murine hematopoietic cells lines, transforms Rat-1 fibroblasts, protects against apoptosis and initiates the sequence of events leading to leukemia in transgenic and retroviral mouse models (reviewed in Elefanty *et al*., 1997).

Since one major consequence of cytokine signalling in normal cells is the induction of cellular differentiation, we wondered whether expression of BCR–ABL could also impact upon this process. It is pertinent that in gene transfer experiments in which murine bone marrow cells were transduced with a BCR–ABL-expressing retrovirus, many of the myeloid tumors which arose in recipient animals displayed a differentiated phenotype, even in the face of the additional oncogenic mutations likely to have occurred during tumor evolution (Daley *et al*., 1990; Elefanty *et al*., 1990; Kelliher *et al*., 1990; Elefanty and Cory, 1992b). Even highly tumorigenic erythroid/megakaryocytic, myeloid and mast cell lines derived from primary BCR–ABL retrovirus-induced tumors continued to exhibit a differentiated phenotype (Elefanty and Cory, 1992a). Similarly, culturing BCR–ABL virus-infected bone marrow progenitor cells in agar in very low concentrations of IL-3 or stem cell factor gave rise to mixed lineage colonies which differentiated along mast cell, macrophage, neutrophil or B lymphoid lineages (Gishizky and Witte, 1992; Skorski *et al*., 1996).

However, there have been no studies which have directly addressed the influence of BCR–ABL expression on myeloid differentiation, although it was notable that a human megakaryoblastic cell line, MO7E, exhibited a more differentiated phenotype after infection with a BCR–ABL-expressing retrovirus (Sirard *et al*., 1994). Indeed, an effect on myeloid differentiation could not have been detected in the extensively studied BCR–ABL transformants of cell lines which have lost their ability to differentiate, such as FDC-P1 and Ba/F3 (Dexter *et al*., 1980; Palacios and Steinmetz, 1985). Therefore, we chose to examine

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the biological effects of BCR–ABL expression in M1 myeloid leukemia cells, a monoblastic line which has retained the ability to terminally differentiate into macrophages in response to the cytokines leukemia inhibitory factor (LIF), interleukin (IL)-6 or Oncostatin-M (Metcalf *et al*., 1988; Shabo *et al*., 1988; Bruce *et al*., 1992).

M1 cells expressing BCR–ABL took on many characteristics of macrophages. However, in contrast to cytokine-differentiated M1 cells, M1.210 cells continued to proliferate, losing the reciprocal relationship usually observed between differentiation and proliferation. These data suggest that BCR–ABL is capable of stimulating pathways of differentiation as well as cellular proliferation. This may play a role in the phenotype observed in chronic myeloid leukemia.

Results

Generation of BCR–ABL-expressing M1 clones

M1 cells were co-transfected with the MPZen(*bcr–abl*) provirus (Hariharan *et al*., 1988) and a puromycin resistance plasmid to obtain M1 cell lines stably expressing BCR–ABL. The MPZen(*bcr–abl*) provirus incorporates the *BCR–ABL* cDNA cloned from the human CML cell line K562 and encodes the 210 kD form of the BCR–ABL protein. Antibiotic-resistant clonal cell lines were assayed for BCR–ABL expression by flow cytometry using an anti-ABL monoclonal antibody (Schiff-Maker *et al*., 1986). This technique has been used by ourselves and others to quantify the level of expression of cytoplasmic proteins (Huang *et al*., 1997; Cambier *et al*., 1998). Multiple puromycin-resistant M1 clonal cell lines expressing BCR–ABL (denoted M1.210) at levels from 5–10-fold higher than the background staining in parental M1 cells were derived from three independent transfections (Figure 1a). Similar phenotypes were displayed by all expressing cell lines surveyed (over a dozen), although detailed analyses were confined to two to five independent clonal cell lines for most experiments. The expected cytoplasmic localisation of the BCR–ABL protein was demonstrated by indirect immunofluorescence (data not shown) and Western blotting indicated that the protein produced was the anticipated size (see Figure 1b for an example). As we had observed previously (Cambier *et al*., 1998), M1.210 cell lines tended to be unstable in continuous culture and many clones lost expression of BCR–ABL over a period of several weeks, even in the presence of antibiotic selection. Therefore, all experiments were performed on recently thawed aliquots of M1.210 clonal cell lines in which BCR–ABL expression had been verified by flow cytometry.

M1.210 clones exhibit macrophage differentiation

Cultures of parental M1 cells consisted of a monomorphic population of moderate-sized, non-adherent blast cells. On the other hand, cultures of M1.210 clones were heterogeneous with many large and semi-adherent cells, reminiscent of the appearance of M1 cultures induced to differentiate along the macrophage lineage by LIF or IL-6. Examination of May–Grünwald–Giemsa stained cytospins revealed that M1.210 cells were large with vacuolated cytoplasm and resembled M1 cells differentiated in LIF for 4 days (Figure 1c). The appearance of M1.210 cells did not change following exposure to LIF. One interesting morphological difference between M1 and M1.210 cells was the high frequency of multinucleated cells in M1.210 clones (from 10–21% of cells in three clonal lines surveyed) compared to the less common binucleated cells seen in LIF-induced cultures of parental M1 cells (approximately 4%). Binucleate M1 cells were restricted to LIF-containing cultures, but multinucleate M1.210 cells were present at approximately the same frequency in unstimulated and LIF-stimulated cultures. Furthermore, mitotic figures were occasionally apparent in the BCR-ABL-expressing multinucleated cells (see Figure 1c for an example) but were not seen in the LIF-treated M1 cultures.

To confirm the morphological impression that BCR–ABL was inducing a differentiated phenotype, we compared the expression of the macrophage cell surface markers F4/80 and $F_c\gamma$RII on the M1.210 clonal cell lines to their expression on undifferentiated and differentiated M1 cells. As shown in Figure 2, unstimulated M1 cells expressed low levels of $F_c\gamma$RII

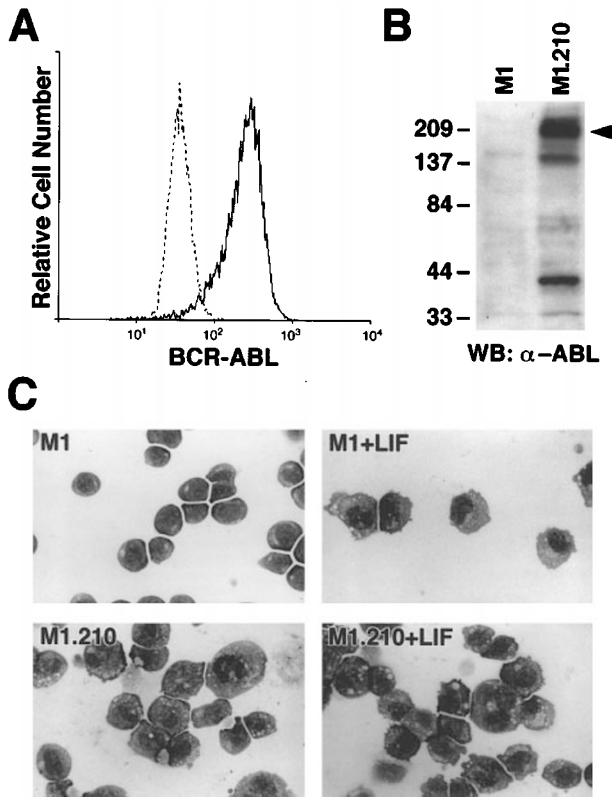

Figure 1 (**a**) Representative intracellular FACS profiles showing BCR–ABL expression detected by an anti-ABL monoclonal antibody (24-21) in M1.210 cells (solid line) compared with the background staining in untransfected parental M1 cells (dashed line). Relative fluorescence values are shown on the X axis. (**b**) Immunoblots of cell lysates from M1 parental or M1.210 cells probed with mouse anti-ABL (α-ABL) monoclonal antibody (8E9). The 210 kD BCR–ABL protein is indicated by the arrowhead. (**c**) May–Grünwald–Giemsa stained cytocentrifuge preparations of unstimulated M1 and M1.210 cells and cultures stimulated for four days in 1000 U/ml of LIF. Several multinucleate cells are seen in the M1.210 and M1.210+LIF panels, including a mitotic cell in the upper right of the M1.210 panel

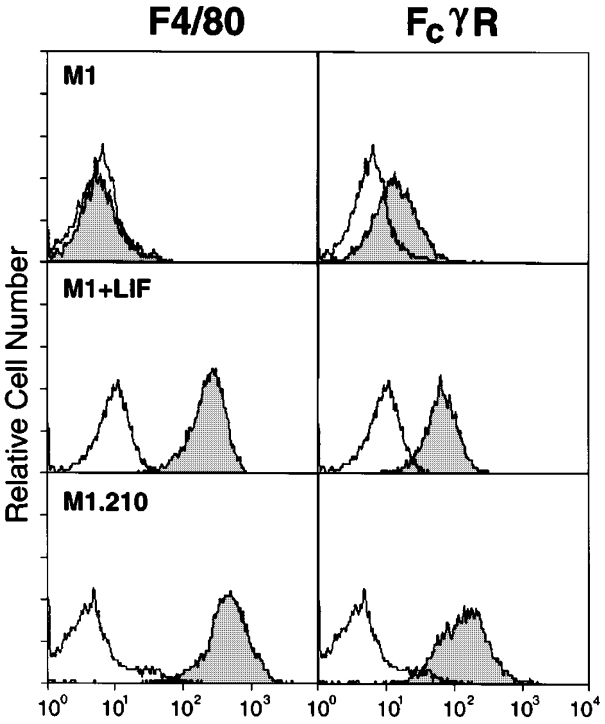

Figure 2 Cell surface expression of macrophage markers detected by antibodies to F4/80 and $F_c\gamma$RII in unstimulated and LIF-treated cultures of M1 cells and in unstimulated cultures of M1.210 cells. Isotype control antibodies are shown as unfilled profiles and specific antibody staining as filled profiles

but not F4/80 and expression of both molecules was increased following four days of LIF-induced differentiation. On the other hand, M1.210 cells constitutively expressed levels of F4/80 and $F_c\gamma$RII that were even higher than those observed on LIF-induced M1 cells.

We examined the phagocytic ability of M1.210 cells to determine whether they had acquired functional characteristics of macrophages (Figure 3). Parental M1 cells did not phagocytose fluorescent latex beads, although beads were occasionally observed attached to the cell membrane (see Figure 3a and b). In contrast, the phagocytic ability of unstimulated M1.210 clonal cell lines (Figure 3d–f) resembled that of the M1 LIF-treated cultures (Figure 3c). The cells were clearly larger than parental M1 cells and many contained one or more phagocytosed latex beads after a 24 h incubation period.

A consequence of macrophage differentiation is the increased expression of cell surface scavenger receptors (MSR) (Kodama *et al*., 1990; Rohrer *et al*., 1990), trimeric integral membrane glycoproteins which mediate the endocytic uptake of oxidised lipoproteins as well as serving as adhesion molecules (Fraser *et al*., 1993). Therefore, we compared the expression of MSR on M1 and M1.210 cells by assessing their ability to take up acetylated low density lipoprotein (LDL). We found that M1.210 cells constitutively expressed MSR, as evidenced by their avid uptake of the fluorescent substrate, diI-labelled acetylated LDL (Figure 4c and d). On the other hand, unstimulated M1 cells did not express functional MSR (Figure 4a) and were only capable of endocytosis of LDL following LIF-induced differentiation (Figure 4b).

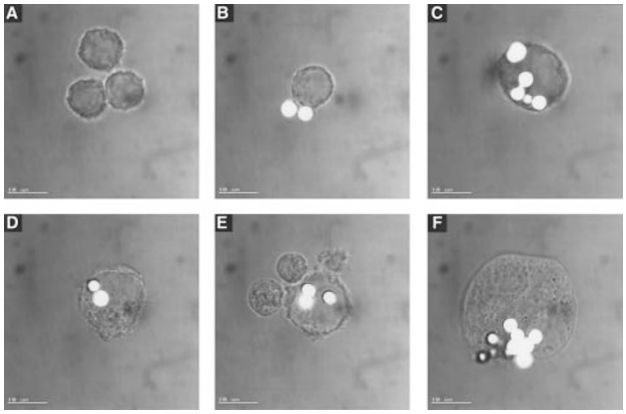

Figure 3 Phagocytosis of fluorescent latex beads by M1 and M1.210 cells. Unstimulated M1 cells (**a, b**) were not phagocytic, although latex beads were occasionally associated with the cell membrane (**b**). M1 cells cultured in LIF for 4 days (**c**) or unstimulated cultures of M1.210 cells (**d–f**) were overtly phagocytic with numerous cells containing one or more intracellular beads. Not all the beads in panel F are in the same focal plane, and several beads are still attached to the cell surface, presumably undergoing phagocytosis. Images are single optical sections captured using a confocal microscope. Comparable phagocytic ability was displayed by two independent M1.210 clones

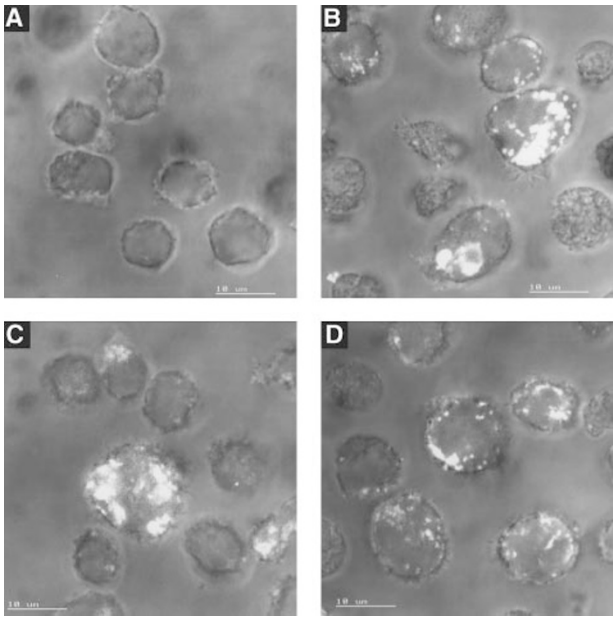

Figure 4 Uptake of diI-labelled acetylated LDL by M1 and M1.210 cells. While M1 cells (**a**) did not express the macrophage scavenger receptor and hence did not take up labelled LDL (seen as white intracellular material) until cultured with LIF (**b**), numerous cells in unstimulated cultures of two independent M1.210 clones took up acetylated LDL (**c,d**). Images are single optical sections captured with a confocal microscope

M1.210 clones are hyporesponsive to LIF or IL-6 but remain partially responsive to dexamethasone

When unstimulated M1 cells are cultured in agar, they form large, compact colonies. However, when cultured in the presence of LIF or IL-6, M1 cells form small, dispersed colonies and clusters and there is loss of clonogenicity (Metcalf *et al*., 1988; Shabo *et al*., 1988). Since M1.210 clones displayed morphological, surface

antigen and functional characteristics in common with cytokine-treated M1 cells, we examined their growth in agar and response to cytokines. The top panel in Figure 5 demonstrates the compact morphology of M1 colonies grown in control agar cultures and shows the small, dispersed colonies developing in cultures containing LIF, IL-6 or the glucocorticoid, dexamethasone. The behaviour of M1.210 clones in agar, however, was very different (Figure 5, second row). In unstimulated cultures, M1.210 colonies were notable for their large size and substantial halo of migrating cells, suggesting enhanced proliferation as well as macrophage differentiation. Furthermore, M1.210 cells demonstrated markedly impaired responses to LIF and IL-6, with little change in colony size or morphology, but remained partially responsive to the growth inhibitory effects of dexamethasone. While the clonogenicity of parental M1 cells was decreased to 40% and 1% of control values when cultured in the presence of LIF and IL-6 respectively, there was no clonal extinction upon culturing M1.210 cells with the same stimuli (Figure 6a). For both parental M1 and M1.210 cells, dexamethasone reduced clonogenic potenial by a similar degree (to 51% and 69%, respectively). The visual impression of larger colony size in M1.210 agar plates was confirmed by comparing the number of cells per colony in unstimulated and stimulated cultures (Figure 6b). M1.210 colonies were approximately three times the size of unstimulated M1 parental colonies and remained unchanged in response to LIF or IL-6 (approximately 12 000 cell/colony), while these cytokines reduced the mean size of M1 colonies from 3400 cells/colony to 40 cells/colony and less than 10 cells/colony, respectively. In contrast to LIF and IL-6, dexamethasone reduced the size of M1.210 colonies (to approximately 6500 cells/colony), though the magnitude of the response (approximately twofold) was still much less than the 20-fold reduction observed in M1 colonies (to approximately 160 cells/colony). The growth rates of M1 and M1.210 cells in liquid culture were consistent with their behaviour in agar. After 1 or 2 days, M1 parental cells grew more slowly in response to IL-6, LIF and dexamethasone (Figure 6c). In contrast, the growth rate of M1.210 cultures was not affected by LIF and was minimally inhibited by IL-6 or dexamethasone (Figure 6d). We excluded the possibility that the lack of response to LIF displayed by M1.210 clones reflected a loss of either the LIF receptor α chain or its heterodimeric partner, gp130, by comparing the binding of radio-iodinated-LIF to M1 and M1.210 clones. Scatchard analysis confirmed that the two M1.210 clonal cell lines tested carried a similar number of high affinity LIF receptors to parental M1 cells (Figure 7).

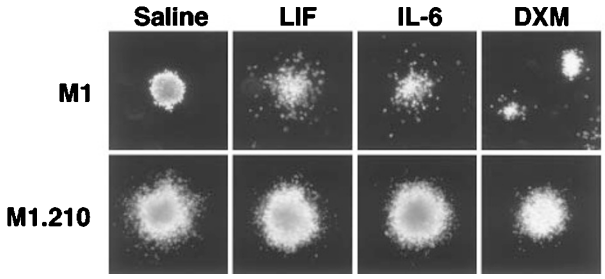

Figure 5 Morphology of colonies of M1 and M1.210 cells cultured in agar in the absence of stimuli (saline) or in LIF, IL-6 or dexamethasone (DXM) for 7 days. Similar morphology was evident in five independent clones

M1.210 cells continue to cycle in the presence of LIF and IL-6

We investigated the impaired LIF and IL-6 responses of M1.210 clonal cell lines by comparing the cell-cycle distribution of M1 and M1.210 cells. The proportion of M1 cells in S phase declined from 44% to 29% or 13% after 4 days' culture in LIF or IL-6 respectively (Figure 8a). There was also an increase in the percentage of cells with a subdiploid ($<2n$) DNA content (from 6% to 68% or 33%), indicative of the apoptosis accompanying the terminal macrophage differentiation induced by these cytokines. Consistent with the high frequency of multinucleate cells in the M1.210 cultures, there was a clearly visible population of cycling cells harbouring a DNA content between 4n and 8n. The cell cycle profiles of M1.210 clones were not influenced by culture in LIF or IL-6. Although the confounding effect of the polyploid subpopulation made it impossible to accurately assess the percentage of cells in each phase of the cell cycle, the percentage of diploid cells in S phase (between 2n and 4n DNA content) remained high ($>60\%$) and the percentage of apoptotic cells was stable (between 4% and 9%). It was also notable that both M1 and M1.210 cells responded similarly to dexamethasone with a prominent G2/M peak and increased apoptosis.

As an independent measure of the proportion of cells in S phase, we compared bromodeoxyuridine (BrdU) incorporation into M1 parental and M1.210 cells cultured with and without LIF for 4 days (Figure 8b). In the absence of LIF, both cell lines incorporated BrdU into a similar proportion of cells (73% of 300 cells counted for M1 and 65% of 300 cells counted for M1.210). While this percentage remained essentially unchanged in M1.210 cells cultured with LIF (68% of 300 cells counted), it fell substantially (to 26% of 300 cells counted) in LIF treated M1 cultures ($P<0.0001$ by χ^2 test). Also, it was only in M1.210 cultures (with or without LIF) that binucleate cells in S phase were observed (Figure 8c).

Expression of MYC in M1.210 cells

It has been shown previously that induction of growth arrest and terminal differentiation by LIF or IL-6 in M1 cells is accompanied by suppression of c-MYC expression (Liebermann and Hoffman-Liebermann, 1989). Therefore, it was of interest to determine whether M1.210 cells still expressed c-MYC despite their differentiated phenotype. As shown in Figure 9, M1.210 cells expressed abundant c-MYC mRNA, at similar levels to unstimulated M1 cells, while c-MYC transcripts were barely visible in LIF-treated M1 cells displaying a comparable degree of differentiation.

Discussion

One of the key features of CML is the near normal myeloid differentiation displayed by the leukemic cells

during the chronic phase of the disease. While this could be interpreted to reflect a minimal interference with normal cellular differentiation by BCR–ABL, an alternative explanation was that induction of differentiation was an integral part of the action of BCR–ABL. To investigate the possible effect of BCR–ABL on myeloid differentiation, we utilised the murine myeloid leukemia cell line, M1, which has been frequently used as a model for macrophage differentiation (Metcalf *et al*., 1988). In much the same way as bone marrow progenitor cells differentiate and eventually stop dividing in response to growth factors, M1 cells respond to various cytokines and glucocorticoids by losing clonogenic potential and undergoing terminal macrophage differentiation. The response is robust and its kinetics have been well studied.

M1 cells were stably transfected with the Zen(*bcr–abl*) provirus (Hariharan *et al*., 1988) and BCR–ABL-expressing clones were identified by flow cytometry. In each of over a dozen independent clones derived from three separate electroporations, BCR–ABL-expressing M1 clonal lines could be

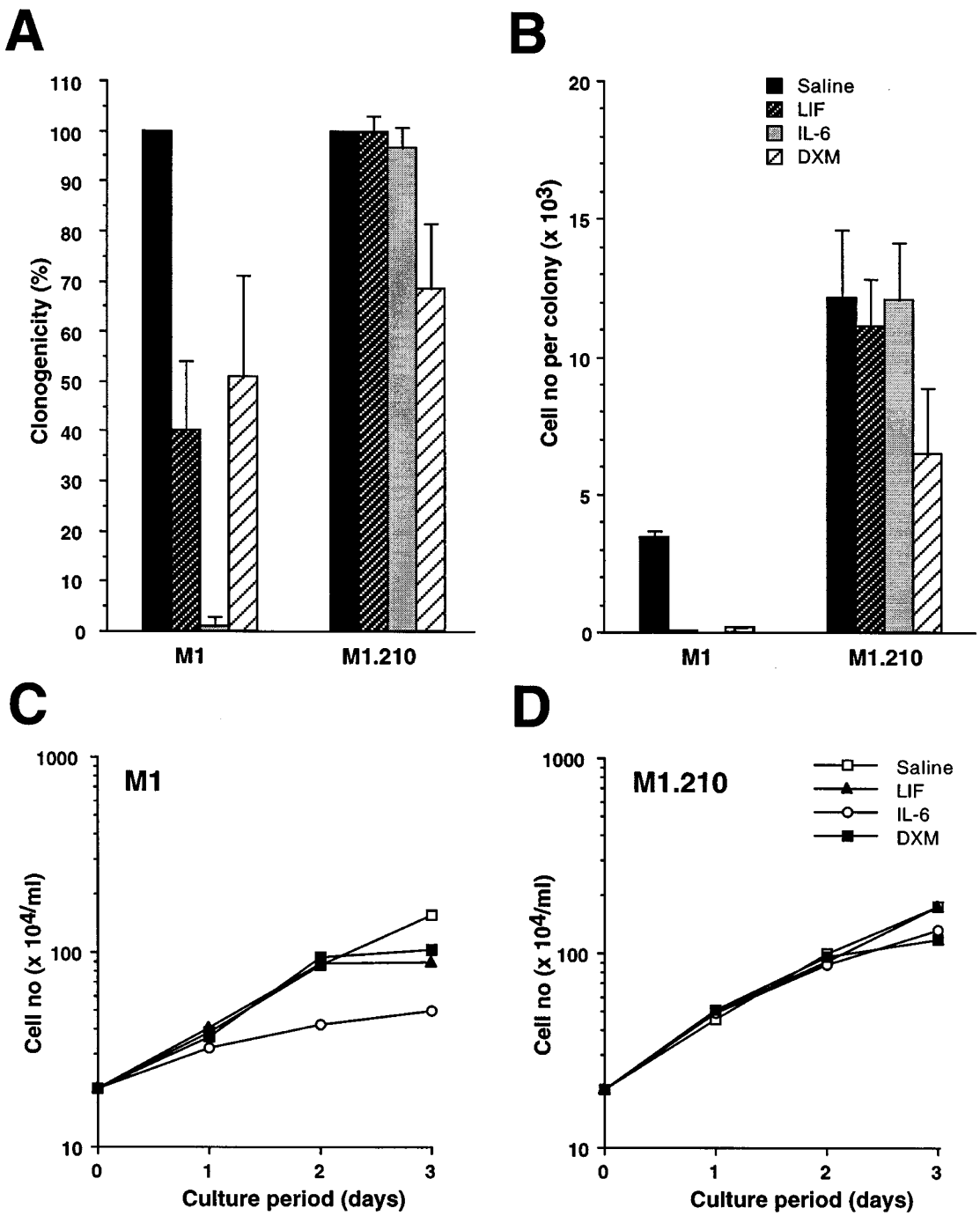

Figure 6 Clonogenicity (**a**) and colony size (**b**) of M1 and M1.210 cells in unstimulated cultures (saline) and cultures treated with LIF, IL-6 or dexamethasone (DXM). The values shown represent the mean ± s.d. of duplicate or triplicate cultures from three experiments comparing M1 cells with a total of five independent M1.210 clonal cell lines. The growth rate of M1 cells (**c**) and a representative M1.210 clonal cell line (**d**) in response to the same stimuli was compared in liquid culture. Each point represents the mean value of duplicate wells

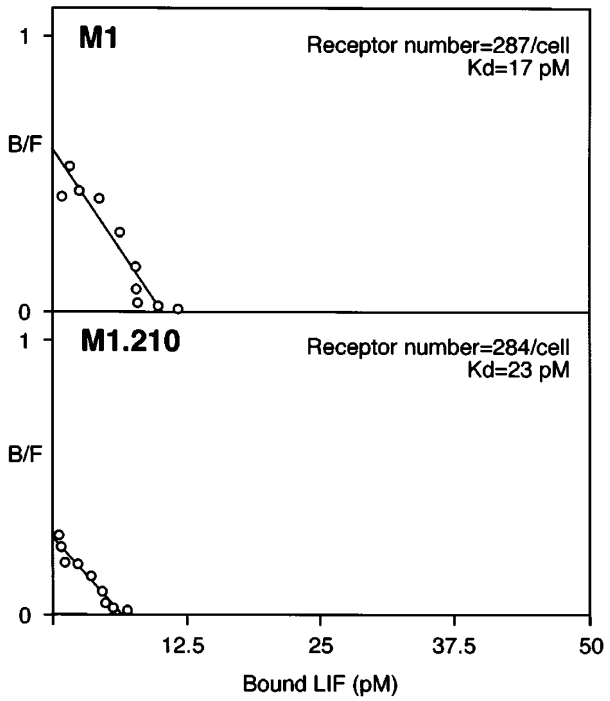

Figure 7 Scatchard analysis of ^{125}I-labelled human LIF (hLIF) binding to M1 parental and M1.210 cells. Cells were incubated with decreasing concentrations of ^{125}I hLIF in the presence or absence of at least 100-fold excess of unlabelled hLIF on ice for 4 h. Bound (B) and free (F) hLIF were separated by centrifugation through fetal calf serum. Scatchard transformation of the data was performed assuming 100% bindability of labelled ligand. Two independent clones of M1.210 cells displayed similar binding characteristics

readily identified in the culture dish by their heterogeneous cell size. Detailed characterisation of M1.210 clonal lines which expressed BCR–ABL at levels 5–10-fold above background, confirmed that they displayed similar morphology, cell surface markers, migration in agar and phagocytic ability to M1 cells cultured for four days in the presence of LIF or IL-6. These data suggested that BCR–ABL was inducing differentiation, perhaps by acting as a surrogate for the JAK family tyrosine kinases normally activated in M1 cells in response to these cytokines (Stahl *et al*., 1994).

An unexpected phenotype endowed upon M1 cells by BCR–ABL was that of persistent proliferation despite a differentiated appearance. In combination with previous studies which showed that BCR–ABL expression led to proliferation of factor-dependent murine hematopoietic cell lines (Daley and Baltimore, 1988; Hariharan *et al*., 1988; Laneuville *et al*., 1991), these data suggested that BCR–ABL activated mitogenic as well as differentiative pathways. In a recent study, Cortez *et al*., (1997) demonstrated that expression of BCR–ABL in growth arrested 32D cells stimulated cells to reenter the cell cycle. This was associated with activation of RAS/MAP kinase pathways (both Erk and JNK), D-type cyclins and cdk2. In fact, previous studies had also identified genes downstream of BCR–ABL such as RAS (Cortez *et al*., 1995, 1996; Tauchi and Broxmeyer, 1995; Elefanty *et al*., 1997), cyclin D1 (Afar *et al*., 1995) and c-MYC (Cleveland *et al*., 1989; Sawyers *et al*., 1992; Afar *et al*., 1994) which were very likely to play a role in proliferation. Recent evidence has linked RAS signalling to cyclin D1/cdk4 activation (Albanese *et al*., 1995; Aktas *et al*., 1997; Peeper *et al*., 1997) and it has been shown that induction of S phase in REF52 cells requires co-expression of both RAS and c-MYC (Leone *et al*., 1997). In keeping with this, we found that M1.210 cells expressed high levels of RAS by immunofluorescence (data not shown) and maintained expression of c-MYC mRNA at levels similar to undifferentiated parental M1 cells.

Another intriguing aspect of the M1.210 cell lines was their refractoriness to LIF- and IL-6-induced growth arrest. This was evident in both liquid and agar cultures and was confirmed by flow cytometric cell cycle analysis and BrdU incorporation. One trivial explanation for the impaired response in M1.210 clones was excluded by the demonstration of normal numbers of high affinity receptors for LIF on the cell surface of M1.210 cells. Comparison of the responses of M1 and M1.210 cells to the glucocorticoid, dexamethasone, revealed that M1.210 cells still remained partially responsive to this agent. This suggested that there were some signalling pathways leading to growth inhibition which were uniquely stimulated by dexamethasone and were not blocked by BCR–ABL. Recent studies using mutants of the granulocyte-macrophage colony stimulating factor receptor β chain have suggested that, in M1 cells, separate signalling pathways lead to suppression of clonogenicity or the acquisistion of a differentiated phenotype (Smith *et al*., 1997). The current data may suggest that BCR–ABL differentially affects these two pathways.

Hyporesponsiveness to LIF and IL-6 is not peculiar to M1.210 cell lines, but is also a feature of M1 cells engineered to constitutively express c-MYB, c-MYC or SCL. These three transcription factors are expressed in unstimulated M1 cells but are normally sequentially silenced within one to two days of cytokine stimulation, prior to growth arrest or the upregulation of molecular or phenotypic markers of macrophage differentiation (Liebermann and Hoffman-Liebermann, 1989; Hoffman-Liebermann and Liebermann, 1991; Resnitzky and Kimchi, 1991; Selvakumaran *et al*., 1992; Tanigawa *et al*., 1995). M1myb cells did not undergo either growth arrest or differentiation upon stimulation by LIF or IL-6 (Selvakumaran *et al*., 1992). In one study, constitutive c-MYC expression substantially blocked the response of M1 cells to LIF or IL-6 (Hoffman-Liebermann and Liebermann, 1991). In the presence of these cytokines, the M1myc cells proliferated at nearly the same rate as untransfected M1 cells and displayed only limited morphological features of differentiation. On the other hand, in a similar study reported by Resnitzky and Kimchi (Resnitzky and Kimchi, 1991), constitutive c-MYC expression in M1 cells led to a very modest perturbation of IL-6-induced responses, with only a slight slowing of IL-6-induced growth arrest and no impairment of differentiation (Resnitzky and Kimchi, 1991). It is possible that these differences are explicable by variability between the M1 sublines used by different workers. Curiously, M1/SCL cells

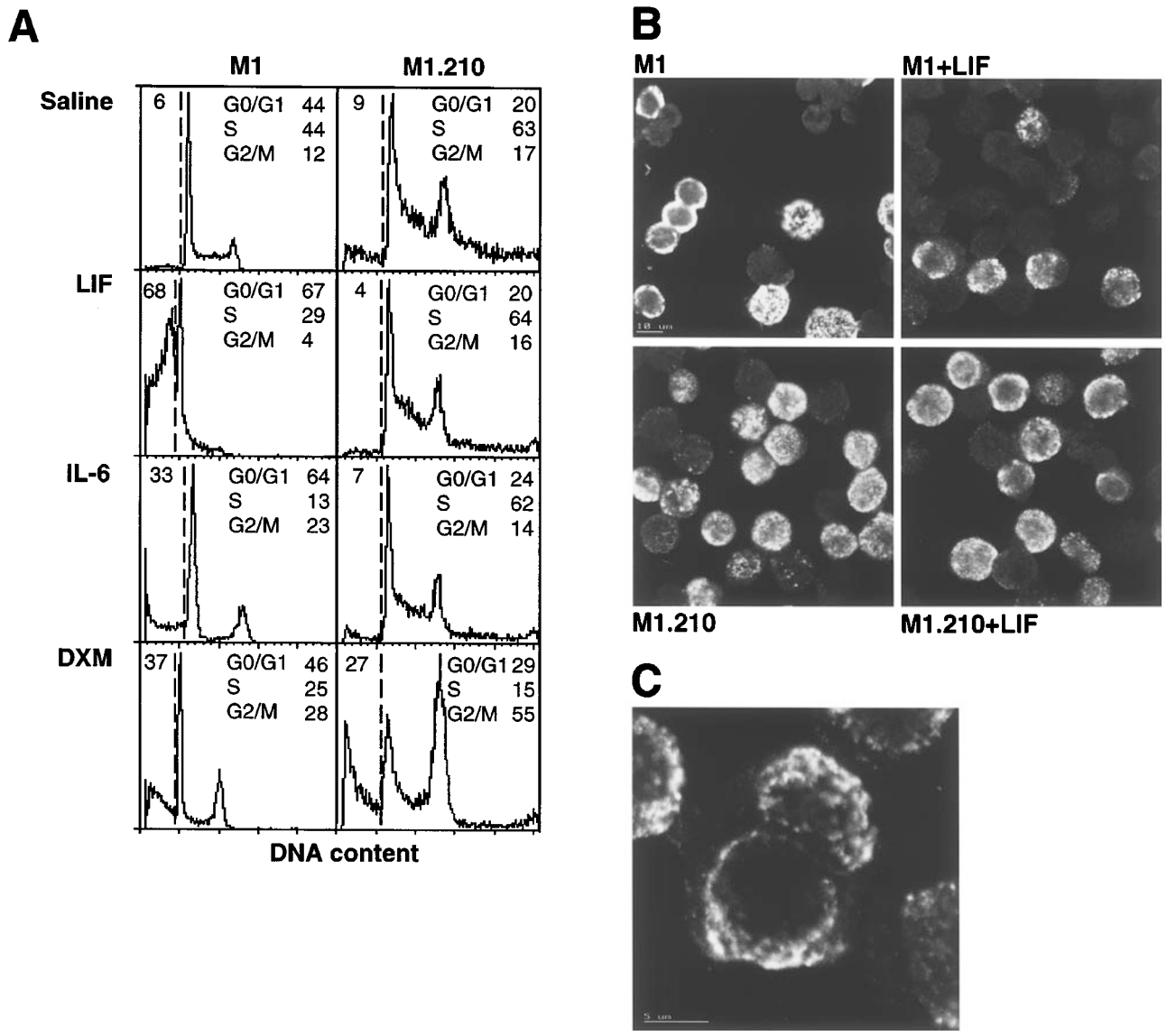

Figure 8 M1.210 cells are refractory to the growth inhibitory effects of LIF and IL-6 but remain partially responsive to dexamethasone. (**a**) Comparison of the cell cycle profiles of M1 and M1.210 cells cultured in the absence of stimuli (saline) or in LIF, IL-6 or dexamethasone (DXM) for 4 days. The percentage of cells with DNA content $<2n$ (assumed to be apoptotic) is indicated to the left of the dashed vertical line. The percentages of viable cells calculated to be in G_o/G_1, S and G_2/M phases of the cell cycle are indicated. The figures are approximate for the M1.210 cultures, since cells with DNA content $>4n$ were not included in the analysis. This experiment was representative of three such experiments, examining a total of eight independent M1.210 clonal lines. In other experiments with M1 cells, the percentages of apoptotic cells present in cultures treated with LIF or IL-6 were more similar. (**b**) M1 and M1.210 cells cultured for 4 days in the presence or absence of LIF were labelled with BrdU for one hour to detect cells in S phase. (**c**) Binucleate cell in M1.210 culture with both nuclei in S phase. Images in (**b**) and (**c**) represent single optical sections captured using a confocal microscope

were partially inhibited in their clonal suppression and differentiation in response to LIF and Oncostatin-M but were indistinguishable from control M1 cultures when exposed to IL-6 (Tanigawa *et al*., 1995). Consistent with their effects on M1 differentiation, constitutive expression of c-MYB and c-MYC also inhibited the differentiation and growth arrest of murine erythroleukemia cells treated with DMSO (Coppola and Cole, 1986; Dmitrovsky *et al*., 1986; Prochownik and Kukowska, 1986; Clarke *et al*., 1988; McClinton *et al*., 1990). Based on these studies, one may speculate that the inappropriately high levels of c-MYC (and c-MYB, data not shown) mRNA expressed by M1.210 cells played a role in inhibiting cytokine-induced growth arrest.

Interestingly, in a study designed to investigate the induction of protein tyrosine phosphatase activity by IL-6 in M1 cells, Zafriri *et al*., (1993) examined two M1 clonal cell lines stably transfected with a *BCR–ABL* expression vector. They did not describe the phenotype of unstimulated M1/BCR–ABL cells, but stated that IL-6 induced growth arrest and differentiation were not prevented by BCR–ABL expression. The reason for the different results in our two studies is unclear, but may reflect variability between the M1 sublines. In this context, it may be relevant that it was the same group who had previously reported the more modest effect of c-MYC expression upon the cellular repsonse of M1 cells to IL-6 (Resnitzky and Kimchi, 1991).

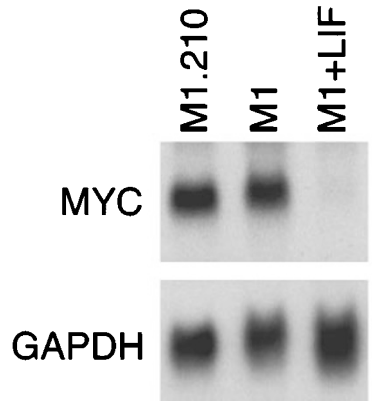

Figure 9 Northern blot analysis of 15 μg of total RNA extracted from M1.210 cells and M1 cells cultured without and with 1000 U/ml LIF (M1 + LIF) for 3 days. The membrane was probed with the c-MYC cDNA. Hybridisation to a probe for GAPDH was used to indicate RNA loading

If the results of our studies are relevant to the phenotype of chronic myeloid leukemia, the expansion of maturing myeloid cells which characterises the chronic phase might reflect the consequences of BCR–ABL-mediated stimulation of multiple signalling pathways impacting upon cell proliferation, differentiation and death, albeit in an abnormal, unbalanced manner. This notion is consistent with the model of 'discordant maturation' proposed by Clarkson and colleagues (see Clarkson *et al*., 1997 for a review). They argue that there may be one or more extra divisions in a primitive progenitor compartment in CML, but that the progenitors are slightly more mature than normal. Also, it is important to bear in mind that differentiation in CML is not completely normal - there is a shift towards immaturity in the myeloid series and asynchronous nuclear/cytoplasmic maturation, basophilia and dysplastic changes including multinucleate erythroblasts are frequently seen. It is tempting to speculate that the multinucleate macrophages produced in our cultures were a reflection of differentiation gone awry. Whilst this may be the case, it seems more plausible that they were indicative of an abnormality affecting cell division, with either a failure of cytokinesis following nuclear division or a more fundamental derangement in negative feedback control of the cell cycle. Pertinent to this latter scenario are the observations that M1 cells are deficient in p53 (Yonish-Rouach *et al*., 1991) and that colon carcinoma cell lines which are deficient in p53 or its downstream target, the p21 cdk inhibitor, undergo additional rounds of S phase without the successful completion of mitosis in response to DNA damaging drugs or irradiation (Waldman *et al*., 1996).

In summary, we have demonstrated that constitutive expression of the BCR–ABL oncogene in M1 myeloid leukemia cells leads to macrophage differentiation without the growth arrest associated with cytokine-induced differentiation. At a molecular level this is likely to reflect the unbalanced stimulation of multiple cytokine signalling pathways, which are yet to be elucidated. Similar events may be responsible for the phenotype in chronic myeloid leukemia.

Materials and methods

Cell lines

M1 cells (Metcalf *et al*., 1988) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal calf serum (FCS). M1 cells were cultured in 1000 U/ml mLIF (AMRAD, Vic), 1000 U/ml mIL-6 (a gift from Drs R Simpson and R Moritz) or 10^{-6} M dexamethasone (Sigma Chemicals, St Louis, MO, USA) for up to 4 days to induce differentiation in liquid suspension culture. BCR–ABL-expressing M1 cell lines (M1.210) were generated by co-transfection of 4×10^6 parental cells with linearised MPZen(*bcr–abl*) retrovirus (Hariharan *et al*., 1988) and puromycin resistance plasmids (a gift from Prof S Cory) in a 10 : 1 molar ratio. A Bio-Rad Gene Pulser (Bio-Rad Laboratories, CA, USA) was used at 270 volts and 960 μF. Transfected cells were diluted post-electroporation into 100 individual wells and selected with puromycin (Sigma Chemicals, St Louis, MO, USA) at 20 μg/ml 48 h after electroporation. Proliferating cells expanded from positive wells were tested for BCR–ABL expression using flow cytometry. Positive cell lines were re-cloned by expanding single colonies grown in soft agar as described (Metcalf, 1984).

Flow cytometry

M1 and M1.210 cells were labelled with biotinylated monoclonal antibodies recognizing F4/80 (Austyn and Gordon, 1981) and $F_c\gamma$RII (Unkeless, 1979) or an isotype control (Pharmingen, San Diego, CA, USA) as described previously (Smith *et al*., 1997). Specific binding was revealed with streptavidin-coupled phycoerythrin (Caltag Laboratories, West Grove, PA, USA). For flow cytometric detection of BCR-ABL, cells were fixed for 10 min in 1% paraformaldehyde in phosphate buffered saline (PBS) at room temperature and permeabilized with 0.3% saponin/PBS (Sigma Chemicals) as described (Strasser *et al*., 1995). The cells were stained by labelling with a monoclonal antibody against ABL (mouse monoclonal 24-21: Oncogene Science Inc., Uniondale, NY, USA) and using an FITC-conjugated goat anti-mouse IgG antibody (Southern Biotechnology, Birmingham, AL, USA) as a secondary reagent. Analyses were performed on a Becton Dickinson FACScan using FACScan software (Becton Dickinson, San Jose, CA, USA).

Agar cultures

M1 and M1.210 clones were assayed for colony forming cells by culturing 100–200 cells/ml in duplicate or triplicate 1 ml agar-medium cultures for 7 days as described previously (Metcalf, 1984). Cultures were stimulated with 1000 U/ml of mLIF, 1000 U/ml mIL-6, 10^{-6} M dexamethasone or saline.

Phagocytic assays

Unstimulated M1 cells, M1 cells pretreated with LIF for 4 days and M1.210 cells were plated at 1×10^5 cells/ml in 24 well dishes and cultured for 24 h with 1×10^6/ml Fluoresbrite 3 μm microspheres (Polysciences Inc., PA, USA). To assess uptake of acetylated LDL, 5×10^5 cells were incubated in 7 μg/ml dil-labelled acetylated LDL (PerImmune Inc., MD, USA) in a total volume of 1.4 ml for 3 h at 37°C. For both experiments, cells were adhered to poly-L-lysine (Sigma Chemicals) coated glass coverslips, fixed with 4% paraformaldehyde/PBS, mounted and analysed using a Leica confocal laser scanning microscope with SCANWARE software (Leica Lasertechnik, Heidelberg, Germany).

Cell cycle analysis

Cell cycle analysis was performed as previously described (Vairo *et al*., 1996). In brief, cells were fixed in 70% ethanol, washed in PBS and incubated in propidium iodide solution (69 μM propidium iodide in 38 mM sodium citrate) containing 5 μg/ml RNase A at 37°C for 20 min. Flow cytometric analysis of DNA content was performed using a FACScan (Becton Dickinson) and cell cycle distribution was determined using the Cell Fit program (Becton Dickinson).

Bromodeoxyuridine labelling of S phase cells

Cells were incubated in medium supplemented with 5 μg/ml 5-bromo-2′-deoxy-uridine (BrdU) (Boehringer Mannheim GmbH, Mannheim, Germany) for 60 min at 37°C. They were washed in PBS, attached to poly-L-lysine (Sigma Chemicals) coated glass coverslips and fixed for 10 min at 4°C in methanol. After rehydration in PBS for 3 min, DNA was denatured by incubating the coverslips in 2 M HCl at 37°C for 60 min. The acid was neutralized by washing twice for 5 min in 0.1 M sodium borate, pH 8.5, followed by three washes of 3 min duration in PBS. Incorporation of BrdU was detected with an anti-BrdU monoclonal antibody (BMC 9318, Boehringer Mannheim) at 6 μg/ml for 45 min at room temperature followed by an FITC-conjugated anti-mouse IgG (Southern Biotechnology, Birmingham, AL, USA) used at 1 : 100 dilution for 30 min. Images were captured as single optical sections on a Leica confocal laser scaning microscope.

Binding studies

M1 parental and M1.210 cells ($1.4-2.0\times10^6$ cells per analysis point) were incubated in duplicate with increasing concentrations of ^{125}I-labelled hLIF with or without unlabelled hLIF (2 μg/ml final concentration) in 100 μl RHF medium (RPMI 1640 medium supplemented with 10 mM HEPES buffer, pH 7.4, and 10% (v/v) FCS) at 4°C for 4 h. Cell-associated and free ^{125}I hLIF were separated by rapid centrifugation through 200 μl FCS and quantified in a Packard γ-counter. Specific binding was determined as the difference in counts between parallel sets of tubes with or without unlabelled hLIF. The data were analysed by the LIGAND program (Munson and Rodbard, 1980) and presented as Scatchard plots (specific bound/free hLIF *versus* bound hLIF).

Western blotting

M1 parental or M1.210 cells (approximately 10^7 per sample) were lysed in buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 2 mM NaF, 1 mM Na_3VO_4, and Complete™ protease inhibitor cocktail (Boehringer Mannheim; one tablet per 50 ml of lysis buffer). After pelleting insoluble material and protein standardisation, the supernatants containing approximately 100 μg of total cellular proteins were electrophoresed under reducing conditions and transferred to polyvinylidene difluoride membranes (PVDF-Plus, Micron Separations Inc., Westborough, MA, USA). After blocking, the membranes were incubated with an anti-ABL monoclonal antibody (clone 8E9; PharMingen, San Diego, CA, USA), followed by a horse-radish peroxidase conjugated goat anti-rabbit IgG antibody (DAKO, Glostrup, Denmark). The BCR–ABL protein was visualised using a chemiluminescent substrate (Pierce Chemical company, Rockford, IL, USA).

Northern analysis

Total RNA was prepared from cultures of M1 and M1.210 cells using Trizol reagent (GIBCO–BRL, Gaithersburg, MD, USA) according to the manufacturer's instructions. Fifteen μg of total RNA was electrophoresed through formaldehyde/agarose gels, transferred to GeneScreen Plus membranes (DuPont, Boston, MA, USA) and hybridized with probes labelled with α-^{32}P dATP using the GIGAprime DNA labelling kit (Bresatec Ltd., Adelaide, South Australia). The probe for c-MYC was a 1.4 kb *Xho*I cDNA fragment and for GAPDH was a 1.1 kb *Pst*I fragment of rat glyceraldehyde-3-phosphate dehydrogenase cDNA.

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