Effect of interleukins on the proliferation and survival of B cell chronic lymphocytic leukaemia cells

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Abstract

Aims—To investigate the effects of interleukin (IL) 1, 2, 4, and 5 on the proliferation and survival of peripheral blood B cells from patients with B chronic lymphocytic leukaemia (B-CLL) and compare them with the effects on normal peripheral blood B cells.

Methods—The proliferation and survival of pokeweed mitogen (PWM) activated B cells from B-CLL (n=12) and normal peripheral blood (n=5) were studied in vitro in response to IL-1, IL-2, IL-4, and IL-5. Survival of cells in cultures with or without added interleukins was studied by microscopic examination of cells and DNA agarose gel electrophoresis.

Results—Proliferation was observed in both B-CLL and normal peripheral blood cells on culture with IL-2 alone and also in some, but not all, B-CLL and normal peripheral blood cells with IL-1 and IL-4. However, there was greater variability in B-CLL cell responses than in normal peripheral blood cells. IL-5 did not affect normal peripheral blood cell proliferation but it increased proliferation in two B-CLL cases. Synergistic effects of these cytokines were not detected. IL-4 inhibited normal peripheral blood and B-CLL cell proliferation after the addition of IL-2. Inhibition of B-CLL cell responses to IL-2 was also observed with IL-5 and IL-1. Survival of B-CLL cells in cultures was enhanced with IL-4 not by an increase in proliferation but by reduced apoptosis. No such effect was seen in normal peripheral blood cells. IL-2 had a less noticeable anti-apoptotic effect; IL-5 enhanced apoptosis in B-CLL cells.

Conclusions—B-CLL and normal peripheral blood cells proliferated equally well in response to IL-2. IL-4 had a much lower effect on B-CLL cell proliferation, but had noticeable antiapoptotic activity. IL-5 enhanced cell death by apoptosis.

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In vivo normal B lymphocytes derive growth factors through interactions with T cells and monocytes.¹ In culture, however, survival and growth of activated B cells depends on the availability of external factors such as in-

terleukin (IL) 4,² IL-2,³ IL-1,⁴ and IL-5.³ These factors could act independently or synergistically in enhancing B cell proliferation.^{3,5}

Chronic lymphocytic leukaemia (CLL) is a neoplastic disease characterised by sustained lymphocytosis with phenotypically mature B lymphocytes inappropriately arrested at an early stage of their programming.⁶ B-CLL cells differ from the majority of morphologically identical, unstimulated normal small B lymphocytes in that they express a pan T cell associated antigen (CD5).⁶ As with normal B lymphocytes, the capacity of activated B-CLL cells to proliferate in response to specific cytokines such as IL-2 and IL-4 has been extensively investigated. A number of investigators claim that B-CLL cells show a proliferative response to IL-2⁷⁻¹⁰ or IL-4.^{7,11,12} Others, however, noted hyporesponsiveness to either IL-2¹²⁻¹⁴ or IL-4.¹⁵⁻¹⁸ Some studies focusing on IL-2^{7,10,11} or IL-4^{7,11} have shown heterogeneity of B-CLL cell proliferative responses to such factors. This may indicate discrete stages of maturation of such cells. Also, in vitro some B-CLL cells may require signals provided by more than one cytokine in order to undergo proliferation.

Alternatively, it is possible that some B-CLL cell populations are unable to survive in culture long enough to respond to the growth factors. Such factors are important for the proliferation and survival of many cell types and, in the absence of these, cells die by apoptosis.¹⁹ Apoptosis is an active process which is important in haemopoiesis not only in the selection of T and B lymphocytes, but also in the generation of myeloid²⁰ and erythroid²¹ cells. This process is also involved in carcinogenesis¹⁹ and is characterised by specific molecular events²² with identifiable morphological and biochemical changes.²³

The concept that different cytokines may have a proliferative role in B-CLL is relatively old, but to some extent still controversial. While some cytokines, namely IL-2 and tumour necrosis factor- α (TNF- α), appear to be involved, the contribution of others (for example, IL-1, IL-4 and IL-5) known to affect growth of normal B cells needs to be clarified. To date, no studies have compared proliferation of B-CLL cells with that of normal B lymphocytes in response to growth factors. Furthermore, examination of the role of these interleukins in cell proliferation and survival may extend our understanding of the disease with implications for treatment.

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Methods

The study population comprised 14 patients with B-CLL, the demographic details of whom are summarised in the table. These patients were not receiving treatment at the time of study and were carefully selected such that more than 50% of their mononuclear cells expressed CD5 and less than 11% expressed CD2. Monoclonality of membrane immunoglobulin was demonstrated on the basis of monotypic κ or λ light chain expression. Healthy volunteers ($n=5$) were picked at random and served as a source of normal peripheral blood B cells.

CELL ISOLATION, PURIFICATION AND IMMUNOPHENOTYPING

Mononuclear cells from heparinised peripheral blood samples from patients with CLL and normal volunteers were isolated by centrifugation on a Ficoll-Hypaque (Nycomed, Sheldon, Birmingham, UK) gradient. After T cell and monocyte separation by positive selection with CD2 coated magnetic beads²⁴ (Dynabeads, Dynal, New Ferry, Wirral, UK) and adherence to plastic (37°C, one hour), respectively. Cell preparations were negative and less than 0.5% positive when stained with the CD3 (Leu2b) and CD14 monoclonal antibodies, respectively, using a standard enzyme immunoassay. For immunophenotyping, a standard indirect immunofluorescence method was used. Fluorescence analysis was performed on a FACSCAN flow cytometer (Becton Dickinson, Mountain View, California, USA).

B CELL PROLIFERATION ASSAY

Cells (2×10^6 /ml) were cultured with or without pokeweed mitogen (PWM) (8 μ g/ml; Sigma, Poole, Dorset, UK) on microtitre plates (Gibco, Paisley, Scotland) in a total volume of 0.2 ml RPMI 1640 culture medium (Gibco) supplemented with L-glutamine (0.3 mg/ml; Gibco), penicillin and streptomycin (100 U/ml and 100 μ g/ml, respectively; Glaxo, Greenford, UK), 10% heat inactivated (56°C, 30 minutes) fetal calf serum (Flow, Herts, UK) and 2-mercaptoethanol (5×10^{-6} M; Sigma). Recombinant IL-1, IL-2 and IL-4 (all purchased from AMS Biotechnologies, Burford, Oxford, UK) were used at 100 U/ml. Human IL-5 was kindly provided by Dr C Sanderson (NIMR,

Mill Hill, London, UK). This sample gave an ED50 of 1 in 6600 in a mouse liquid eosinophil differentiation factor (EDF) marrow assay which equated to about 1 in 400 000 for a human liquid EDF assay. Interleukin-5 was used at $100 \times$ ED50 for the human liquid EDF assay. The cells were cultured at 37°C for three days in a humidified atmosphere of 5% CO₂ in air, pulsed with 1 μ Ci/well ³H-thymidine (specific activity 5.0 Ci/ml; Amersham, Bucks, UK) for a further two days before being harvested on to glass fibre disks with a Diatech multiple cell harvester. The cell associated radioactivity was measured by a standard liquid scintillation counter. Each sample was cultured five times and the results were expressed as mean counts/culture.

EFFECT OF CYTOKINES ON CELL SURVIVAL

B cells ($1-2 \times 10^6$ cell/ml) from B-CLL ($n=4$) and normal ($n=4$) peripheral blood were cultured in medium at 4°C (control)²⁵ or at 37°C (experimental) with or without cytokines. At specific time intervals, cells (200 μ l aliquots) were stained with acridine orange (5 μ g/ml; Sigma) for five minutes before the total number of stained cells and cells with visibly condensed chromatin (apoptotic) was obtained using fluorescence microscopy. At least 400 cells were counted from different fields taken at random.

DNA EXTRACTION AND AGAROSE GEL ELECTROPHORESIS

Cells (2×10^6 cell/ml) were lysed for four to five hours at 37°C in Tris-EDTA buffer (50 mM Tris, 10 mM EDTA; pH 8.0) containing 0.5% sodium dodecyl sulphate (SDS; Sigma) and ribonuclease (0.5 mg/ml; Sigma). The mixture was treated with proteinase K (0.5 mg/ml; Sigma) before the DNA was extracted twice with equal volume of phenol chloroform isoamyl alcohol (25:24:1 v/v, respectively; Sigma). After the addition of 0.1 volume of 3 M sodium acetate (pH 4.5; Sigma), the DNA was precipitated with two volumes of absolute alcohol at -20°C overnight followed by centrifugation for 10 minutes at 6500 rpm in a microcentrifuge. The precipitated DNA was redissolved in Tris-EDTA buffer (10 mM Tris, 1 mM sodium EDTA; pH 8.0) overnight at 4°C. DNA samples were elec-

Immunohaematological data of the patients with B-CLL

Patient	Immunophenotypes*								
	WBC ($\times 10^9$ /l)	CD2	CD5	CD20	FMG7	CD10	CDHLA	κ	λ
1	11	5	94	58	5	<1	81	<1	<1
2	25	2	50	66	24	<1	55	<1	<1
3	27	8	73	5	<1	<1	67	<1	<1
4	30	11	85	40	1	<1	77	46	10
5	23	2	89	28	<1	<1	83	<1	<1
6	43	7	80	39	<1	1	40	2	<1
7	26	9	52	39	10	<1	56	1	<1
8	11	2	89	28	<1	<1	83	<1	<1
9	39	1	70	44	NT	1	78	3	34
10	13	9	78	62	3	<1	65	NT	NT
11	20	5	91	38	2	<1	85	<1	<1
12	3	6	80	59	5	<1	73	70	10
13	27	12	66	56	23	<1	21	80	8
14	132	2	98	75	1	3	87	5	9

* Percentage of positive cells determined by immunofluorescence; NT = not tested; WBC = white blood cells. Samples from patients 13 and 14 were used for DNA agarose gel electrophoresis.

Figure 1 Proliferation of B-CLL cells (from 12 patients) and normal peripheral blood cells (from five subjects) in response to IL-1, IL-2, IL-4, and IL-5, added either singly or in combination. Cells were cultured with PWM and the appropriate concentration of interleukin(s) for five days before proliferation was measured as a function of ^3H -thymidine incorporation. Each sample was cultured in pentaplicate and the results are expressed as mean counts/culture (mean + SEM).

trophoresed on a 1.8% agarose gel in Tris-borate buffer (0.045 M Tris, 0.5 M boric acid, 1 mM EDTA) containing 0.5 µg/ml ethidium bromide (Sigma) and photographed under ultraviolet illumination.

STATISTICAL ANALYSIS

One way analysis of variance was used to investigate whether there was a significant difference between leukaemic and normal cell response to treatment (growth factor). The homogeneity of variances was analysed using Bartlett's test. Paired *t* test was used to analyse the effect of growth factor on either B-CLL or normal peripheral blood cell proliferation.

Results

EFFECT OF CYTOKINES ON B-CLL AND NORMAL PERIPHERAL BLOOD CELL PROLIFERATION

The effect of adding cytokines to the culture medium on B-CLL and normal peripheral blood cell proliferation is presented in fig 1. Interleukin-2 had a significant proliferative effect in all controls and in 10 of 12 B-CLL cases; the mean (range) percentage increase in proliferation was 173% (108–252%) ($p < 0.01$) and 458% (197–976%) ($p < 0.001$), respectively. Interleukin-1 and IL-4 had a variable

effect; with IL-1, proliferation increased in 80% (four of five) of controls by a mean of 84% (41–125%) ($p < 0.02$) and by 192% (72–405%) in four of 12 B-CLL cases ($p < 0.001$). Similarly, IL-4 enhanced cell proliferation by 116% (85–172%) in 80% of controls ($p < 0.02$) and by 235% (123–400%) in four of 12 B-CLL cases ($p < 0.001$). Interleukin-5 had no effect on normal peripheral blood cells ($p > 0.1$) and increased proliferation in only two of 12 B-CLL cases by a mean of 284% (184–386%). One way analysis of variance showed no significant differences between normal peripheral blood and B-CLL cell responses to any of the growth factors (range of *p* values: 0.126–0.990). However, B-CLL cell responses to all cytokines alone were more variable than those of the normal peripheral blood cells as revealed by analysis of homogeneity of the variances of the normal peripheral blood and B-CLL cell responses using Bartlett's test (range of *p* values: 0.021–0.089). None of the cytokines acted synergistically with regard to normal peripheral blood and B-CLL cell proliferation (fig 1). However, IL-4 significantly inhibited the response of normal peripheral blood ($p < 0.02$) and B-CLL ($p < 0.001$) cells to IL-2 (fig 1). Interleukin-1 also significantly inhibited IL-2 induced proliferation in B-CLL ($p < 0.02$) and normal peripheral blood ($p < 0.05$) cells.

SURVIVAL OF B-CLL AND NORMAL PERIPHERAL BLOOD CELLS AFTER CULTURE WITH CYTOKINES

In longstanding liquid culture with PWM, B-CLL cells survived better in the presence of IL-4 than with any of the other cytokines (fig 2) as revealed by morphological apoptotic changes in acridine orange stained cells ($p < 0.05$; day 6). Interleukin-2 had a less noticeable antiapoptotic effect (fig 2) which was not significant ($p > 0.1$; day 6). With IL-5, apoptosis increased during the first few days in culture (fig 2; IL-5, $p < 0.02$ on day 1 of culture). Addition of IL-1 had no significant effect on apoptosis. On day 8 of culture, the mean

Figure 2 Percentage fall (mean) in apoptosis on addition of IL-1, IL-2, IL-4, and IL-5 on given days after culture at 37°C. The difference is between percentage apoptosis in the absence and presence of cytokine ($n = 4$ for IL-2, IL-4 and IL-5; $n = 3$ for IL-1).

Figure 3 Proliferation of B-CLL cells in the presence or absence of IL-2 or PWM. Cells were cultured in pentaplicate for two or five days before ³H-thymidine incorporation was measured. Results are presented as mean + SEM of three different experiments; patients 3, 9 and 10; $p < 0.01$.

(range) percentage of remaining cells showing condensed chromatin and/or apoptotic bodies was 69% (48–82%) ($n=4$) with no interleukins, 59% (39–69%) ($n=4$) with IL-2 alone, 48% (34–72%) ($n=4$) with IL-4, 65% (47–77%) ($n=3$) with IL-1, and 73% (65–83%) ($n=4$) with IL-5. The mean (range) of percentages of apoptosis of control cultures (that is, at 4°C) were as follows: with no interleukin, 3.9% (3.5–4.2%) ($n=4$); with IL-2, 4.0% (2.5–5.1%) ($n=4$); with IL-4 3.9% (2.9–4.1%) ($n=4$); with IL-1, 5.3% (4.5–6.2%) ($n=3$); and with IL-5, 4.4% (3.5–5.2%) ($n=4$).

Lack of mitogenic activation did not affect survival patterns of B-CLL cells in response to any of the interleukins (results not shown). The

antiapoptotic activity of IL-4 was not the result of early proliferation of cells in response to this growth factor as revealed by ³H-thymidine incorporation experiments. However, B-CLL survival in the presence of IL-2 was most likely due to increased cell numbers as a result of cell proliferation (fig 3); proliferation with IL-2 was significantly increased in three cases studied on days 2 and 5 (fig 3; $p < 0.01$). DNA agarose gel electrophoresis also showed that cells cultured at 4°C alone or with IL-4 or dexamethasone (positive control) did not undergo apoptosis (fig 4). It also confirmed that IL-4 protects against B-CLL cell death in culture as the intensity of the bands of DNA extracted from cells cultured with IL-4 was significantly weaker than that in the absence of IL-4 (fig 4). Furthermore, DNA electrophoresis revealed that IL-5 enhances apoptosis of cultured B-CLL cells; the intensity of DNA extracted from cells cultured with IL-5 was significantly stronger than that found in the absence of IL-5 (fig 5). In contrast to B-CLL cells, the percentage normal peripheral blood cells undergoing apoptosis did not decrease on culture with IL-4.

Discussion

These results confirm the previously reported observations that IL-2 enhances normal peripheral blood³²⁶ and B-CLL⁷¹⁰ cell proliferation. As reported before, IL-4 increased proliferation of both normal peripheral blood²²⁷ and B-CLL⁷¹⁰¹¹ cells. However, enhanced proliferation on culture with IL-4 was observed in fewer B-CLL cells. Although production of IL-1 by B-CLL (and normal peripheral blood)

Figure 4 Agarose gel electrophoresis of DNA extracted from B-CLL cells after incubation with or without IL-4 or dexamethasone. Lane 1, without IL-4 at 37°C; lane 2, with IL-4 at 37°C; lane 3, without IL-4 at 4°C; lane 4, with IL-4 at 4°C; lane 5, DNA size marker (BstE λ digest); lane 6, with dexamethasone at 37°C; lane 7, with dexamethasone at 4°C. [DNA]/well, 2 μ g in lanes 1, 2, 5, 6, and 7; 1.5 μ g in lanes 3 and 4. Lanes 1 and 2, apoptotic DNA equivalent to 49 and 27%, respectively.

Figure 5 Agarose gel electrophoresis of DNA extracted from B-CLL cells after incubation for two days at 37°C with or without IL-5. Lane 1, no additions; lane 2, with IL-5. Percentage apoptosis: lane 1, 56%; lane 2, 77%.

cells is a well known phenomenon,²⁸ there have been no reports on the effect of recombinant IL-1 on B-CLL cells. This study reports significant proliferation of B-CLL (and normal peripheral blood) cells in response to IL-1. Again, this effect was observed in fewer B-CLL cells. By contrast, Ugglä *et al*²⁹ reported that B-CLL cells do not proliferate in the presence of B-CLL culture supernatant fluids with IL-1-like activity.

There have been no reports on the effect of IL-5 on B-CLL B cell (that is, CD5 positive cells) proliferation although the murine cytokine has been shown to be a growth factor for CD5 positive lymphoma cells.³⁰ In addition, in normal peripheral blood cells, IL-5 does not affect proliferation directly but is thought to enhance expression of IL-2 receptors³ making the cells more responsive to IL-2. However, in this study IL-5 and IL-2 did not act synergistically in either of the cell types studied. On the contrary, IL-5 significantly inhibited the response of the B-CLL cells to IL-2. As, in this study, IL-5 enhanced B-CLL cell death by apoptosis, inhibition of IL-2 induced proliferation by IL-5 may have been the result of increased cell death induced by IL-5 and not because of a direct effect on the response to IL-2. Furthermore, this study confirmed that IL-4 inhibits IL-2 induced proliferation in normal peripheral blood^{31,32} and B-CLL^{16,33} cells. The response of B-CLL and normal peripheral blood cells to IL-5 and IL-1 was also inhibited. No significant differences were observed in B-CLL or normal peripheral blood cell proliferation induced by any of the cytokines, either alone or in combination. The variable B-CLL cell responses to IL-2^{7,10,11} or IL-4^{7,11} have been reported before and have been mainly attributed to the variable B-CLL cell maturity and defective expression of receptors for growth factors.^{12,15} B-CLL cell antigenic immaturity may also explain the hyporesponsiveness of these cells to IL-2¹²⁻¹⁴ and IL-4.¹⁵⁻¹⁸

Survival of B-CLL cells in long term culture, as measured by the rate of apoptosis was noticeably enhanced in the presence of IL-4. Cells also seemed to survive better in the presence of IL-2. However, this enhanced cell survival in the presence of IL-2 was probably the result of proliferation. Interleukin-1 had no effect on B-CLL cell survival. By contrast, IL-5 seemed to enhance cell death by apoptosis, especially in the first few days of culture.

The apoptotic activity of IL-5 and the anti-apoptotic function of IL-4 on B-CLL cells in vitro are interesting findings and require further investigation. Recently, Dancescu *et al*³⁴ showed that IL-4 protected B-CLL cells from spontaneous and hydrocortisone induced apoptosis. Panayiotidis *et al*³⁵ also reported enhanced B-CLL survival in vitro in the presence of IL-4 and attributed this effect to inhibition of DNA fragmentation (apoptosis) by IL-4. Dancescu *et al*³⁴ also showed that IL-1, IL-2 and IL-5 did not have any protective effect against hydrocortisone induced apoptosis of B-CLL cells. By contrast, we found that IL-5 enhanced spontaneous apoptosis of B-CLL cells. How IL-5 induces apoptosis of B-CLL

cells and how IL-4 protects such cells is unknown. Generally, very little is clear about the regulation, biochemical mechanisms and identity of the genes or their products which regulate apoptosis.³⁶

In conclusion, IL-1, IL-2, IL-4, and IL-5 induced a similar response in PWM activated B-CLL and normal peripheral blood cells. The responses of B-CLL cells to growth factors were significantly more heterogeneous than those of normal peripheral blood cells, probably the result of B-CLL surface antigenic immaturity. Survival of B-CLL cells in long term cultures was greatly enhanced by IL-4, an effect not seen in normal peripheral blood cells. Spontaneous apoptosis of B-CLL cells was not affected by IL-2 but was enhanced by IL-5. This study supports previous reports on the effect of some cytokines on B cell proliferation, but also extends our understanding of the proliferative and apoptotic responses of B-CLL cells which may enable the development of new therapeutic strategies.

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