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Increased spontaneous secretion of IL-6 from B cells of patients with B chronic lymphatic leukaemia (B-CLL) and autoimmunity

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SUMMARY

We studied B cells from 18 patients with B-CLL, six of them with autoimmune haemolytic anaemia, for spontaneous secretion of IL-6. Our aim was to determine whether the increased incidence of autoimmune disease found in B-CLL patients is associated with enhanced spontaneous IL-6 secretion. IL-6 was measured by the effect of B cell supernatants on the proliferation of an IL-6 dependent plasmacytoma cell line T1165. The highest IL-6 values ($7{\cdot}4 \pm 1{\cdot}8$ U/ml) were measured in supernatants derived on day 3 of culture from lymphocytes of the six patients with B-CLL and concomitant autoimmune disease. The maximal IL-6 values for 10 patients with B-CLL only were $2{\cdot}8 \pm 0{\cdot}3$ U/ml and for 10 age-matched controls, $0{\cdot}8 \pm 0{\cdot}3$ U/ml ($P < 0{\cdot}01$, each group compared with the other). We conclude that there is an association between B-CLL, autoimmune disease and the spontaneous *in vitro* secretion of IL-6. Further studies are needed to determine whether the IL-6 secretion plays a role in the pathogenesis of autoimmune disease in patients with B-CLL.

Keywords IL-6 secretion chronic lymphatic leukaemia and autoimmunity

INTRODUCTION

Patients with B-CLL have an increased frequency of autoimmune disorders such as autocytotoxicity to haematopoietic cells, systemic lupus erythomatosus (SLE) and autoantibodies (Siegal, 1987; Chikkappa, Zarrabi & Tsan, 1986; Gale, 1987). The mechanism(s) underlying the autoimmune phenomena in B-CLL are uncertain. T cell defects may lead to uncontrolled proliferation of B cell subsets involved in response to autologous antigens. Alternatively, B-CLL malignant cells may provide an antigenic stimulus to the normal residual B or T cells with a resultant cross-reactive attack on haematopoietic cells (Gale, 1987). It has also been speculated that a lack of the normal feedback loop provided by anti-idiotypic antibodies leads to the emergence of autoantibody specificities that would otherwise be neutralized (Bussel, Morell & Skvaril, 1986; Rossi, Sultan & Kazatchkine, 1986; Siegal, 1987).

It has recently been suggested that the lymphokine IL-6 may be a principal lymphokine for autoimmune disease (Kishimoto, 1987). IL-6, previously called B cell stimulatory factor 2 (Kishimoto, 1987), 26-kD protein (Haegeman *et al.*, 1986), interferon-beta (Zilberstein *et al.*, 1986), hybridoma–plasmacytoma growth factor (Aarden *et al.*, 1987) or hepatocyte-stimulating factor (Siegal, 1987), is a multi-functional cytokine produced by lymphoid and non-lymphoid cells (Sporn & Roberts, 1988). IL-6 induced the final maturation of normal, activated B cells to antibody-forming cells. When produced in elevated amounts by lymphocytes from SLE patients (Gaspar, Alvarez-Mon & Gutierrez, 1988; Tanaka *et al.*, 1988), or by tumour cells (Kishimoto, 1987) it could be responsible for autoimmune phenomena and autoantibody production.

Here we hypothesized that production of IL-6 by malignant B-CLL lymphocytes may play a role in the increased incidence of autoimmune diseases found in such patients. We studied serum concentration and the spontaneous secretion of IL-6 by B cells derived from 18 patients with B-CLL without autoimmunity, six B-CLL patients with autoimmunity, and 10 age-matched controls.

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SUBJECTS AND METHODS

Patients

The study group consisted of 18 patients seen in the Haematology Clinic, Lady Davis Carmel Hospital, with B-CLL. They included nine women and nine men, aged 60–82 years. All had $> 15\,000$ lymphocytes/mm^3 during at least a 6-month period. Four patients had lymphocytosis only, seven had accompanying lymphadenopathy, and the other seven patients had both lymphadenopathy and splenomegaly. Six patients were considered to have accompanying autoimmune disease. They had anaemia, elevated reticulocyte counts and a positive direct Coombs test. One patient had transient maculopapular rash,

and also fever with no documented source during haemolysis. Three patients had thrombocytopenic purpura, one of them 6 months before anaemia developed. One patient had transient rash including palms and soles, and arthralgia. One patient had Raynaud's phenomenon. None of the patients had received any treatment 1 month prior to testing.

Preparation of B cells

Mononuclear cells were isolated from heparinized peripheral blood from healthy volunteers and patients with B-CLL by using Leucosep tubes (Assaf Pharmaceutical Industries, Jerusalem, Israel) and Ficoll–Hypaque (Pharmacia, Uppsala, Sweden). The recovered cells were washed with phosphate-buffered saline (PBS) and resuspended at a concentration of 5×10^6/ml in RPMI 1640 medium (Biological Industries, Beit Haemek, Israel) containing 10% fetal calf serum (FCS) (Biological Industries), 100 U/ml penicillin and 100 μg/ml streptomycin. The cell suspension was incubated in plastic culture dishes (Nunc, Roskilde, Denmark) at 37°C for 1 h in 5% CO_2 and 95% air. Non-adherent cells were removed by three gentle washes with PBS and incubated with AET-treated sheep erythrocytes in RPMI 1640 with 10% FCS for 1 h at 4°C and rosette-forming cells were depleted by Ficoll–Hypaque centrifugation. The non-rosette-forming cells were then treated with 1/20 diluted anti-monocyte monoclonal antibodies and anti-T cell monoclonal antibodies (anti-Leu M3 and anti-CD3; Becton Dickinson, Mountain View, CA) for 1 h at 4°C and then with rabbit complement (Cedarlane Lab.) at 37°C for 1 h. The recovered cells were used as purified B cells. In the controls, this cell population contained >95% surface-immunoglobulin positive cells and less than 0·5% Leu M3$^+$ or CD3$^+$ cells. In the B-CLL patients the cell fraction contained 40–80% surface-immunoglobulin-positive cells and less than 0·5% monocytes and T cells.

Preparation of culture supernatants

B cells (10^6/cc) were cultured in RPMI 1640 containing 10% FCS and antibiotics at 37°C, 5% CO_2 and 95% air, in 24-well culture plates for 1–7 days. The culture supernatant was recovered by centrifugation (400 *g* for 10 min) after 1, 3, 5 and 7 days and stored at −70°C until assayed.

T1165 plasmacytoma cell line

The T1165 plasmacytoma cell line, induced originally in a Pristane-conditioned BALB/c mouse was established *in vitro* by Nordan *et al.* (1986). T1165 cells secrete IgA myeloma protein and require for *in vitro* growth the so-called plasmacytoma growth factor (PGF) elaborated by the murine P338D$_2$ macrophage cell line. The T1165 cell line was kindly provided by Professor M. Revel (Weizmann Institute of Sciences, Rehovot, Israel). The cells were maintained in RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, 100 μg/ml penicillin and 100 μg/ml streptomycin, 5×10^{-5} M 2-mercaptoethanol and 10% conditioned medium of P338D$_1$ cell cultures. To prepare the conditioned medium, confluent monolayers of P338D$_1$ cells were cultured for 5 days in RPMI 1640 with 2 mM L-glutamine. The addition of LPS (5 μg/ml) purified from *Escherichia coli* (Sigma Chemical Co., St Louis, MO) significantly increased the production of PGF.

The conditioned medium was filtered through a 0·45 μm syringe filter before addition to the T1165 cell cultures.

T1165 cell proliferation assay

Prior to assay, exponentially growing T1165 cells were washed twice in culture medium without PGF; they were then seeded at a density of 1×10^4 cells/well with test samples, diluted in 100 μl of culture medium without PGF in 96-well flat-bottomed tissue culture plates. Alternatively, recombinant IL-6 (Amgen Biologicals, Thousand Oaks, CA) 1–10 U/ml, recombinant IL-1 (Cistron, Pine Brook, NJ) 1–10 U/ml, recombinant tumour necrosis factor (TNF) (Amgen Biologicals) 1–200 ng/ml, IL-2 1–10 U/ml (Sigma) and BCGF (Sera Lab.) 10% v/v, were added to the wells. Serum from controls, 8-CLL patients without autoimmunity and B-CLL patients with autoimmunity was added to the cells in 1/5 dilution, alone or together with known recombinant IL-6 amounts. After 24 h of incubation at 37°C, 1 μci of ^{3}H-thymidine (Amersham International, Amersham, UK) in 20 μl of medium was added to each well and the cells were harvested 24 h later with the aid of a semi-automatic Linca cell Harvester (Linca, Israel). Results are expressed in U/ml of IL-6. To the supernatants which were found to contain stimulation activity, a 1/10 000 dilution of rabbit anti-human polyclonal IL-6 (Genzyme, Boston, MA) was added, and the supernatants were retested.

Statistical analysis

The B-CLL patients were divided into two groups: those with and those without autoimmune disease. Student's *t*-test was used to test for significant differences between means. For non-parametric data, Fisher's exact test was used. $P < 0.05$ was considered significant.

RESULTS

The maximal spontaneous secretion of IL-6 from B cells cultured at 10^6 cells/ml occurred on days 1 and 3 with a drop off on days 5 and 7 (Table 1). On days 1, 3 and 5, supernatants derived from B-CLL patients with autoimmunity had significantly increased IL-6 values compared with those of CLL patients without autoimmunity ($P < 0.002$).

During the whole culture period patients with B-CLL, with and without autoimmunity, revealed significantly higher IL-6 values than controls ($P < 0.01$). Whereas all the patients with B-CLL and autoimmunity had IL-6 values higher than 5 U/ml, only one of the 12 patients with B-CLL only had comparable IL-6 values (4 U/ml) and two had IL-6 values of 3·5 U/ml and 3·1 U/ml.

In order to correlate to the *in vivo* stimulation, IL-6 concentration was also measured in serum of patients and controls. Significantly ($P < 0.001$) higher IL-6 values were found in the serum of B-CLL patients with B-CLL and autoimmunity compared with those B-CLL patients and controls. No difference was found in serum concentration of IL-6 between B-CLL patients without autoimmunity and controls (Table 1). However, the levels of serum IL-6 were lower than those obtained in B-cell supernatants. We therefore measured the effect of different sera with known IL-6 concentrations on the proliferation of T1165 cells to 10 U/ml recombinant IL-6 (Table 2). All sera were inhibitory (also on the basal proliferation of the cell line, as can be seen in Table 3). It seems that the same magnitude of inhibition was present in all types of serum (Table 2).

The addition of IL-1 and TNF did not significantly increase ^{3}H-thymidine uptake into T1165 cells (Table 3), whereas BCGF and IL-2 caused some degree of proliferation.

Table 1. Serum concentration and secretion of IL-6 by B cells of B-CLL patients and healthy controls (mean ± s.e.m.)

Group	IL-6 (U/ml) Day 1	Day 3	Day 5	Day 7	Serum (20% concentration)
B-CLL with autoimmunity ($n=6$)	7·1 ± 1·6*†	7·4 ± 1·8*†	4·6 ± 0·7*†	3 ± 0·3†	4·0 ± 1·0*‡
B-CLL without autoimmunity ($n=12$)	2·7 ± 0·3†	2·8 ± 0·6†	2·2 ± 0·3†	2·2 ± 0·5†	0·6 ± 0·4
Normal controls ($n=10$)	0·8 ± 0·2	0·8 ± 0·3	0·6 ± 0·2	0·7 ± 0·3	0·5 ± 0·4

B cells derived from autoimmune B-CLL patients, B-CLL patients without autoimmunity and controls were incubated for 7 days. Supernatants were collected after 1, 3, 5 and 7 days and tested for their ability to enhance the proliferation of T1165 cells.

* Significantly ($P<0·002$) higher IL-6 values in autoimmune B-CLL patients compared with non-autoimmune CLL patients.

† Significantly ($P<0·01$) higher IL-6 values in non-autoimmune and autoimmune B-CLL patients compared with normal controls.

‡ Significantly ($P<0·001$) higher IL-6 values in autoimmune CLL patients compared with non-antimmune B-CLL patients and normal controls.

Table 2. Effect of 20% serum on the response of T1165 to recombinant IL-6

Group	Concentration IL-6 in serum added	Expected IL-6 (U/ml)	Measured IL-6 (U/ml)
B-CLL with autoimmunity	5·1	15·1	8·9
	3·7	13·7	7·2
	4·1	14·1	7·5
B-CLL without autoimmunity	0·3	10·3	4·3
	1·2	11·2	4·2
	0·2	10·2	4·7
Normal controls	0·5	10·5	6·2
	0·8	10·8	4·4
	0·2	10·2	4·7

Recombinant IL-6 (10 U/ml) was added together with 20% sera of known IL-6 activity to T1165 cells. The expected IL-6 values were always higher than those measured.

Table 3. The effects of cytokines on the proliferation of T1165 cells

Cytokines added	Proliferation (ct/min)
PGF (1/10 dilution)	18340 ± 1942
rIL-6 (10 U/ml)	21212 ± 1614
rIL-6 (5 U/ml)	15103 ± 358
rIL-6 (2·5 U/ml)	7483 ± 512
rIL-1 (10 U/ml)	2412 ± 344
TNF (200 ng/ml)	2166 ± 657
IL-2 (10 U/ml)	4115 ± 616
BCGF (1/10 dilution)	5269 ± 798
—	2187 ± 380
Normal serum (20%)	752 ± 126

Plasmacytoma growth factor (PGF) was added in 1/10 dilution which was found optimal for T1165 cell line growth. The proliferation of T1165 cells was measured by the uptake of ^{3}H-thymidine. r, recombinant.

The addition of PGF or IL-6 resulted in dose–response proliferation of the T1165 cells. The addition of antibodies to IL-6 or to positive supernatants resulted in reduced ^{3}H-thymidine uptake into T1165 cells (Table 4).

DISCUSSION

The results presented here demonstrate that B cells derived from B-CLL patients with autoimmune manifestations spontaneously secrete high amounts of biologically identified IL-6. B cells derived from patients with B-CLL with no autoimmunity also secrete IL-6, albeit in lower quantities.

T cells and monocyte contamination in the purified B cell population did not exceed 1%, and when cultured alone they did not produce IL-6. Therefore, CLL B cells, the majority of which constitute the malignant clone, are most probably the IL-6 secretors.

Activated B cells, B cell lines and freshly isolated B-CLL cells have recently been found to produce IL-1 (Morabito *et al.*, 1987), TNF (Cordingley *et al.*, 1988) and B cell growth factors (Gordon *et al.*, 1986; Kawamura *et al.*, 1986; Lemoine *et al.*, 1988; Rossi *et al.*, 1988). It was therefore necessary to confirm that our assay was indeed specific for IL-6. It should be noted that there is a controversy concerning the specificity of T1165 cells for IL-6: whereas Le, Reis & Vilcek (1988) had found that these cells proliferate in response to IL-1 and TNF in addition to IL-6, Nordan *et al.* (1986) reported that T1165 cells did not proliferate when IL-1 was added. In our assay, T1165 cells did not respond to IL-1 and TNF but proliferated vigorously to recombinant IL-6. Furthermore, the addition of anti-IL-6 antibodies to IL-6 or to positive supernatants markedly reduced T1165 cell measured IL-6 in the B cell supernatants.

Recently, Freeman *et al.* (1989) observed IL-6 gene expression in several B cell malignancies, but not in B-CLL. In contrast, Biondi *et al.* (1989) found that B cells from a significant proportion of B-CLL patients constitutively express IL-6 gene and secrete variable amounts of the biologically active protein. Our results confirm and extend these latter findings.

Table 4. The effect of antibodies to IL-6 on the response of T1165 cells to supernatants with known IL-6 levels

Source	IL-6 content (U/ml)	After addition of anti-IL-6 (1/10000, $2 \cdot 10^{-4}$ ng/ml)
Recombinant IL-6	10 (21212)	1·5 (1605)
	5 (15103)	1·1 (980)
	2·5 (7483)	0·8 (479)
Supernatant B-CLL with autoimmunity	11·7 (24894)	0·9 (630)
	4·1 (13217)	0·3 (261)
	7·5 (16914)	0·6 (394)
Supernatant B-CLL without autoimmunity	4 (13000)	1·1 (980)
	3·1(9750)	0·8 (479)
	1·9 (2607)	0·6 (396)

Supernatants or recombinant IL-6 were incubated with anti-IL-6 for 24 h at 4°C and then added to T1165 cells. Anti-IL-6 itself did not affect the basal proliferation of T1165 cells (not shown). The results in parenthesis are shown in ct/min.

The biological significance of IL-6 secretion in B-CLL patients with and without autoimmunity is not clear. It is tempting to speculate that autoimmunity in patients with B-CLL is related to the degree of spontaneous IL-6 secretion. However, it should be remembered that B cells from autoimmune patients can produce and respond to a wide variety of factors, which were not measured in this study. Also, B cells derived from CLL patients with no evidence of autoimmunity were able to secrete IL-6, thus suggesting that additional factors are needed to explain autoimmune phenomena in B-CLL. IL-6 is a B cell growth and differentiation factor and may play a role in autocrine or paracrine circuits of immunoregulation. In plasmacytomas, SLE and different tumours, autocrine and paracrine loops involving IL-6 have been demonstrated (Kishimoto, 1987; Kawano *et al.*, 1988; Gasper *et al.*, 1988; Tanaka *et al.*, 1988). Such loops have not been found in B-CLL. *In vitro*, B-CLL cells have little if any proliferative activity and it is very difficult to drive them to differentiate (Hivroz *et al.*, 1986; Sauerwein, van der Meer & Aarden, 1987; Carlsson *et al.*, 1988). The high secretion of IL-6 during the days 1–3 of the culture, and the decline towards the days 5 and 7, are in accordance with an *in vivo* secretion, stimulated by an unknown trigger which is lost in culture. Significantly higher serum IL-6 concentration was indeed found in patients with B-CLL and autoimmunity, compared with the two other groups. Serum inhibitors probably caused disappearance of the difference between B-CLL patients without autoimmunity and controls, and reduction in all IL-6 values. Measuring IL-6 values in supernatants could therefore point to the difference between B-CLL patients with and without autoimmunity and to B cells as the source of the interleukin.

Since the autoimmune manifestations were not uniform and the number of patients was small, it was impossible to correlate severity of the autoimmune disease and IL-6 production. Further studies are needed to measure IL-6 production *in vivo* and to establish the possible relationship between its secretion and autoimmune diseases or other clinical manifestations of B-CLL.

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