

## INTERLEUKIN 4 COUNTERACTS THE INTERLEUKIN 2-INDUCED PROLIFERATION OF MONOCLONAL B CELLS

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IL-4 or B cell stimulating factor is a polypeptide that was originally described, in murine models of B cell response, as a costimulant with anti-IgM antibody for the entry of resting B cells into the S phase of the cell cycle (1). Recent studies have shown that IL-4 is associated with multiple biologic activities. IL-4 increases the expression of class II MHC molecules on resting B cells (2, 3) and promotes IgG1 and IgE production by LPS preactivated B cells (4, 5). Although initially believed to be principally active on B cells, IL-4 has been shown to act on normal T cells (6, 7), T cell lines (7-9), mast cell lines (10), and hematopoietic lineage cells (11, 12).

Recently, the isolation of a cDNA sequence coding for human IL-4 has been reported (13). Human IL-4 is able to promote the growth of activated normal B cells (14), of activated T cells (15), and to induce the expression of CD23 antigen on normal B cells (16).

Monoclonal lymphocytes from B-type chronic lymphocytic leukemia (B-CLL)<sup>1</sup> patients can respond to some growth factors (17, 18). The analysis of this responsiveness is important to define the pattern of reactivity of these malignant cells to physiological signals. In addition, the use of monoclonal B cells frozen at a given stage of differentiation and possibly arising from a discrete B cell subset may help in the understanding of the effects of interleukins. This work was designed to define the effects of IL-4 on these B-CLL lymphocytes. We thus studied the effects of IL-4 on a panel of B lymphocytes from 12 B-CLL patients. In no instance did IL-4 synergize with anti- $\mu$  antibody to support DNA synthesis. Moreover IL-4 profoundly suppressed the response to IL-2 in the 10 patients responsive to this interleukin. Thus, IL-4 may have an inhibitory effect on the proliferation of selected B cell populations.

### Materials and Methods

*Patients.* 12 patients with hematological diagnosis of chronic lymphocytic leukemia were studied. They were not treated at the time of blood drawing, except patient 11, whose lymphocytes were studied at several times before and after initiation of treatment with identical results. The patients' mononuclear cells were >80% positive for the CD19, CD20, and CD5

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<sup>1</sup> Abbreviation used in this paper: B-CLL, B-type chronic lymphocytic leukemia.

antigens and <10% positive for the CD3 antigen. When membrane Ig could be detected, monoclonality could be demonstrated on the basis of monotypic light chain expression.

**Reagents.** mAbs mouse mAb OKT3 (anti-CD3 antigen) and OKM5 were purchased from Ortho Diagnostic Systems Inc. (Raritan, NJ). The B1 and B4 mAb (anti-CD20 and CD19 antigens, respectively) were obtained from Coulter Immunology (Margency Andilly, France). The Leu-1 mAb (anti-CD5) was from Becton Dickinson & Co. (Mountain View, CA); the IOT14 (anti- $\alpha$  chain of the IL-2-R CD25) was provided by Immunotech (Marseille Luminy, France). mAb25 (19) (anti-CD23 antigen) was used at 5  $\mu$ g/ml final dilution. Insolubilized rabbit antibody specific for human  $\mu$  chain (anti- $\mu$  antibody) was purchased from Bio-Rad Laboratories (Richmond, CH). FITC-conjugated F(ab')<sub>2</sub> fragment of goat anti-mouse Ig was from Tago Inc. (Burlingame, CA).

**Factors.** rIL-2 was a gift of Biogen (SA, Geneva, Switzerland). The preparation used had an estimated activity of 10<sup>7</sup> U/mg protein and contained 97.3% IL-2 by SDS-PAGE analysis. IL-4 was obtained from Dr. K. Arai (DNAX Research Institute, Palo Alto, CA) as supernatants from L cells transfected with the peD or PEBT vector containing the human cDNA clone (13). Some experiments were performed with highly purified L cell-derived IL-4. A unit of IL-4 was defined as described previously (14). We verified the biological activity of these IL-4 preparations on normal human B cells; when used at 50–100-U/ml concentrations, they proved able to induce the expression of the CD23 marker, and to enhance DNA synthesis in the presence of anti- $\mu$  antibody (data not shown). A mock preparation consisting of culture supernatants of L cells transfected with a nonrelated cDNA was also used. Recombinant human IFN- $\gamma$  was obtained from Roussel Uclaf (Romainville, France). The preparation had a specific activity of 10<sup>7</sup> U/mg protein.

**Cell Preparations.** B-CLL cells were purified as follows. Mononuclear cells were isolated from the blood of B-CLL patients by centrifugation on a Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) gradient. T cells were eliminated by two cycles of rosetting using aminoethylisothiuronium bromide-treated sheep red blood cells. These preparations were analyzed using a FACS TAR flow cytometer. They were homogeneous small cells, contained <0.5% cells labeled by the OKT3 and by the OKM5 antibodies, and were devoid of activation markers (17).

**B Cell Proliferation Assay.** Cells (10<sup>5</sup>) were cultured in flat-bottomed microtest tissue culture plates (Falcon Labware, Oxnard, CA) in 0.2 ml RPMI-1640 culture medium supplemented with 1% L-glutamine, 1% antibiotics, 2-ME (0.5  $\times$  10<sup>-5</sup> M), and 2% FCS. The B cell growth was assayed in two different systems. In the one-step assay, B cells were cultured for 3 d with anti- $\mu$  antibody (7  $\mu$ g/ml) in the presence of IL-2 or IL-4. In the two-step assay, B cells were cultured alone for 24 and 48 h with IL-2, IL-4, and with both interleukins. Cells were extensively washed and cultured with factors for 3 additional days. Cells were pulsed with 0.5  $\mu$ Ci of [<sup>3</sup>H]thymidine (CEA, Saclay, France) during the last 16 h of culture period, and incorporation of [<sup>3</sup>H]thymidine was measured by a standard liquid scintillation counter. Cultures were performed in duplicate and the results were expressed as the mean of counts per minute per culture. All experiments were performed at least two times, and data shown were from one representative experiment.

**Fluorescence Analysis.** Cells were cultured for 24 h with various factors and washed twice before staining. 0.5  $\times$  10<sup>6</sup> viable cells were treated with antibody at the appropriate dilution in PBS containing 2% BSA and a 0.1% sodium azide (PBS/BSA/azide). After a 30-min incubation at 4°C, the cells were washed twice in cold PBS/BSA/azide, resuspended in 50  $\mu$ l of 1/10 dilution of FITC-conjugated goat F(ab')<sub>2</sub> anti-mouse Ig, and incubated for 30 min at 4°C. Thereafter, the cells were washed twice in PBS/BSA/azide and resuspended in PBS/2% formaldehyde. Fluorescence analysis was performed on a FACS TAR flow cytometer.

## Results

**IL-4 Has No Growth Activity on B-CLL Cells.** B cells from 12 B-CLL patients were costimulated with anti- $\mu$  antibody and IL-2 or IL-4. The cells from 10 of these 12 patients were able to respond to IL-2 by an enhanced DNA synthesis. In no instance

TABLE I  
*IL-4 Does Not Support DNA Synthesis by B-CLL Lymphocytes*

Interleukins <sup>§</sup>	Response (cpm of patient number)*:					
	1 <sup>†</sup>	2	3 <sup>†</sup>	4 <sup>†</sup>	5 <sup>†</sup>	6 <sup>†</sup>
Medium	305	339	584	519	2,789	622
IL-4: 100 U/ml	446	586	ND	ND	2,104	ND
50 U/ml	395	639	1,083	1,569	1,991	295
10 U/ml	476	860	989	1,314	2,818	332
5 U/ml	358	781	934	1,957	3,725	402
IL-2: 50 U/ml	25,379	1,078	24,872	42,202	26,243	1,130
10 U/ml	19,983	1,042	17,414	31,602	17,741	899
Mock: 10% vol/vol	458	689	983	ND	ND	510
5% vol/vol	432	432	732			432

\* The response was assessed on day 3 and expressed as cpm.

<sup>†</sup> In these experiments purified IL-4 was used.

<sup>§</sup> Cells were cocultured with anti- $\mu$  antibody (7  $\mu$ g/ml) and the indicated amounts of interleukins, expressed as final U/ml for IL-4 and IL-2 and as final dilutions for mock (these dilutions corresponded to that of 50 and 10 U/ml of IL-4, respectively).

did IL-4 stimulate DNA synthesis in these conditions. These negative results were obtained with IL-4 concentrations up to 100 U/ml. Data from six representative patients among responders and nonresponders to IL-2 are presented in Table I. The same data were obtained when the response was measured on day 6 (data not shown). In another series of experiments, B-CLL cells from three patients were preincubated with anti- $\mu$  antibody for 2 d, separated from the insolubilized anti- $\mu$  antibody, and cultured for 3 additional days with the same interleukins. Again, IL-4 did not enhance DNA synthesis. The response of anti- $\mu$ -activated cells was  $1,180 \pm 237$  cpm in the presence of 100 U/ml of IL-4 as compared with  $858 \pm 170$  cpm in the absence of interleukin. The same anti- $\mu$ -activated cells did respond to IL-2:  $17,327 \pm 2,545$  cpm. In one additional patient we verified that a 2-d preculture of B cells with IL-4 (50 U/ml) did not enhance the response to anti- $\mu$  antibody in a second-step incubation (data not shown).

*IL-4 Inhibits the Response to IL-2.* We then tested whether IL-4, although devoid

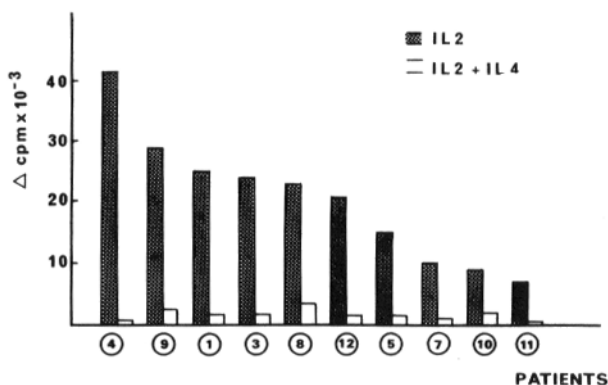


FIGURE 1. IL-4 profoundly suppresses the response to IL-2. In the presence of anti- $\mu$  antibody cells were cultured with IL-2 (50 U/ml), in the absence or in the presence of IL-4 (50 U/ml). Cultures from patients 1, 3, 4, and 5 were performed with purified IL-4.  $\Delta$  cpm was calculated on day 3.

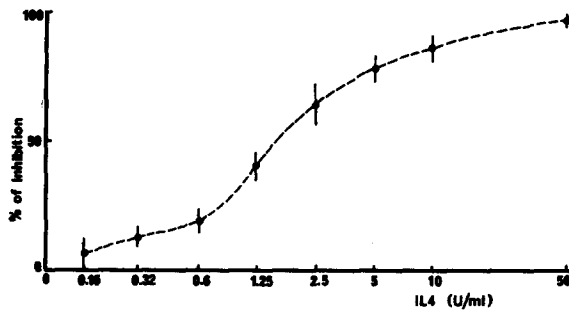


FIGURE 2. Dose-effect curve of IL-4 on the response to IL-2. B-CLL cells from three patients were cultured in the presence of anti- $\mu$  antibody + 50 U/ml of IL-2, in the absence of IL-4, or in the presence of graded concentrations of IL-4. Results are expressed as percent inhibition by a given concentration of IL-4 (mean  $\pm$  SEM from three patients). The percent of inhibition was calculated as follow: Percent inhibition =  $1 - [\text{cpm}(\text{cells} + \text{IL-4} + \text{IL-2}) - \text{cpm}(\text{cells} + \text{IL-4})] / [\text{cpm}(\text{cells} + \text{IL-2}) - \text{cpm}(\text{cells})]$ . The responses in the absence of IL-4 (expressed as  $\Delta$  cpm) were: 21,866, 33,312, and 30,240.

of effect in itself, might interfere with the responsiveness of B-CLL lymphocytes to IL-2. Cells from the 10 patients responding to costimulation by anti- $\mu$  antibody and IL-2 were selected. They were cultured for 3 d with anti- $\mu$  antibody and IL-2 (50 U/ml) in the absence or presence of 50 U/ml of IL-4 (Fig. 1). In all instances, IL-4 profoundly inhibited the response to IL-2 (mean suppression:  $90 \pm 2\%$ ). These results were obtained with a purified preparation of IL-4 in four patients (patients 1, 3, 4, and 5) as well as a crude preparation (the six other patients). The mock preparation had no inhibitory effect in the six patients tested (mean suppression: 3%).

In three patients (patients 3, 4, and 9) the dose-effect curve of IL-4 was established. B-CLL lymphocytes were stimulated with anti- $\mu$  antibody and 50 U/ml of IL-2, and the effects of various concentrations of IL-4 were determined (Fig. 2). The 50% inhibitory concentration of IL-4 corresponded to 1.5 U/ml. An identical result was obtained when the inhibitory effect of IL-4 was assayed in the presence of 5 U of IL-2 (data not shown).

In the same experiments DNA synthesis was measured on day 6 in order to verify that IL-4 would not delay the response to IL-2. IL-4 inhibited the day 6 response to the same extent as the day 3 response (data not shown). It should be pointed

TABLE II  
*IL-4 Suppresses the Response to IL-2 Alone*

Patients	DNA synthesis		
	IL-4	Medium	IL-2
1*	-	871	3,152
	+	653	654
8*	-	548	5,998
	+	441	1,337
10	-	779	7,592
	+	860	1,712

Cells were cultured with IL-2 (50 U/ml) (without anti- $\mu$  antibody) and in the absence or presence of IL-4 (50 U/ml). Results were expressed as cpm on day 3.

\* Purified IL-4 was used.

TABLE III  
*IL-4 Inhibits the Response to the Combination of IL-2 and IFN- $\gamma$*

Factors	DNA synthesis	
	IFN- $\gamma$	Anti- $\mu$ antibody
Medium	490	470
IL-2 (50 U/ml)	31,388	7,422
IL-4 (50 U/ml)	489	384
IL-4 + IL-2	815	592

Cells from patient 11 were cultured in the presence of IFN- $\gamma$  (1,000 U/ml) or anti- $\mu$  antibody (7  $\mu$ g/ml). DNA synthesis was measured on day 3. Purified IL-4 was used.

out that IL-4 profoundly suppressed the response to IL-2, regardless of its magnitude, even when this response was low (see Fig. 1). In the same line we verified that IL-4 did not enhance the response to costimulation with anti- $\mu$  antibody and IL-2 in cells from patients unresponsive to IL-2. The cells from patients 2 and 6 (see Table I) were costimulated with anti- $\mu$  antibody and IL-2 in the absence or presence of IL-4. Their marginal response to IL-2 was not enhanced by IL-4 (data not shown).

*The Inhibitory Effect of IL-4 Is Observed in the Absence of Anti- $\mu$  Antibody.* The previously described experiments were performed in the presence of anti- $\mu$  antibody as this reagent is required to obtain an optimal response to IL-2 in most cases of B-CLL. However, in some cases IL-2 alone is able to support DNA synthesis. The cells from three patients meeting this criteria were selected in order to examine the interaction between IL-2 and IL-4 in the absence of other stimulants. Cells from these patients were stimulated with IL-2 alone (50 U/ml) in the absence or presence of 50 U/ml of IL-4. As shown in Table II, IL-2 did stimulate DNA synthesis in these cells and this response was fully inhibited by IL-4. In the same conditions the supernatants of 10-d cultures from these patients were examined for the presence of Ig. In no instance was Ig detected, regardless of the presence of IL-4. We verified that IL-4 did not affect cell viability. At the end of a 6-d culture of B-CLL cells, the recovery of viable cells (evaluated by Trypan blue exclusion) was determined and expressed as percent of the initial cell input. In the presence of IL-2 (50 U/ml) alone

TABLE IV  
*Expression of CD25 and CD23 Antigens*

Interleukins*	Cells expressing: <sup>†</sup>	
	CD25	CD23
	%	
Medium	12.0	5.6
IL-2	30.6	2.7
IL-4	15.4	11.9
IL-2 + IL-4	32.9	10.9

\* Cells from patient 8 were cultured for 24 h with: medium, 50 U/ml of IL-2, 50 U/ml of purified IL-4, or with both interleukins.

<sup>†</sup> Cells were washed and positivity was assessed by indirect immunofluorescence and flow cytometry.

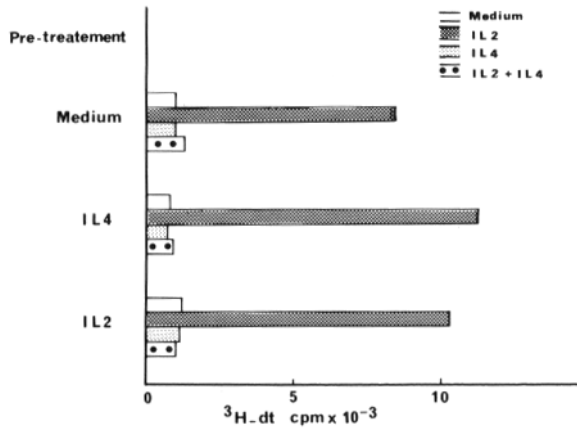


FIGURE 3. The simultaneous presence of IL-2 and IL-4 is necessary for inhibition. Cells from patient 8 were pretreated for 48 h (the y axis) with medium, 50 U/ml of IL-2, or 50 U/ml of purified IL-4. Cells were washed and cultured for 3 additional days. Cultures were performed with IL-2 (50 U/ml), IL-4 (50 U/ml), or both interleukins.

the recovery of viable cells was 73%, to be compared with 80% in the presence of IL-2 (50 U/ml) and IL-4 (50 U/ml).

*IL-4 Inhibits the Costimulation by Interferon- $\gamma$  and IL-2.* We previously showed (20) that IFN- $\gamma$ , although in our hands unable to support DNA synthesis by B-CLL cells, can in some patients enhance the responsiveness to IL-2. We thus tested whether IL-4 could inhibit the costimulation of B-CLL cells by IL-2 and IFN- $\gamma$ . In the presence of IFN- $\gamma$  (1,000 U/ml) or anti- $\mu$  antibody, B-CLL cells were cultured with IL-2, IL-4, and with both interleukins for 3 d. As shown in Table III, in the presence of IL-4 the response to IL-2 and IFN- $\gamma$  was suppressed to the same extent as that to IL-2 and anti- $\mu$  antibody.

*IL-4 Can Deliver a Positive Signal to B-CLL Cells.* One of the most clear-cut early effects of IL-4 on resting B lymphocytes is the upregulation of the expression of CD23 antigen (16). We thus wondered whether IL-4 was able to exert such an effect on B-CLL cells. The cells from patient 8 were selected as they expressed a low, and potentially upregulation-susceptible, percentage of CD23<sup>+</sup> cells (Table IV). A 24-h incubation of these cells with IL-4 alone induced a twofold increase in the number of CD23<sup>+</sup> cells. This effect of IL-4 was observed regardless of the presence of IL-2. Thus, these two interleukins did not interact for the expression of CD23. Similarly IL-4 did not affect the enhancement of CD25 antigen by IL-2. The cells from patient 8 were able to respond to IL-2 in the absence of anti- $\mu$  antibody (see Table II). We could thus verify that both effects of IL-4 (on CD23 expression and on the response to IL-2) could take place in the same monoclonal cells. After preincubation with IL-4, these cells were extensively washed and cultured with IL-2 alone or in the concomitant presence of IL-2 and IL-4 (Fig. 3). Again, IL-4 suppressed the response to IL-2. It should be pointed out that the continuous presence of IL-4 was required for this inhibition to take place: cells pretreated by IL-4 and cultured with IL-2 alone were fully able to respond to this interleukin. We also verified that pretreatment with IL-2 did not prevent the suppressive effect of IL-4 (Fig. 3, bottom).

### Discussion

IL-4 is an interleukin with pleiotropic effects, acting on multiple targets among immunocompetent cells (21). Whether used alone or in combination with another

signal, IL-4 is able to positively affect several aspects of the B cell response. In view of the heterogeneity of the B cell response patterns to IL-4, we decided to analyze the effects of this interleukin on monoclonal B cells, obtained from B-CLL patients. These cells can respond to several interleukins. They are considered as frozen at a given differentiation step and could thus exhibit a dissociated responsiveness to some of the effects of IL-4. They probably arise from a discrete subpopulation of normal B cells of which they may be representative.

One of the most conspicuous effects of IL-4 on human as well as murine B cells is the enhancement of anti- $\mu$ -induced DNA synthesis (1, 14). In a first series of experiments we tested whether this effect could be demonstrated on B-CLL cells. Negative results were obtained with cells from 12 different patients even at IL-4 concentrations up to 100 U/ml. We verified that this lack of response was not due to a delayed peak of DNA synthesis and was not associated with a terminal differentiation. This unresponsiveness was apparent in two-step incubation experiments, regardless of the reagent used initially. It could not be attributed to the inefficiency of the signal provided by anti- $\mu$  antibody, as cells from 10 patients did respond to costimulation with IL-2 and anti- $\mu$  antibody. Thus, in contrast to normal polyclonal B cells, B-CLL cells do not respond to anti- $\mu$  antibody and IL-4 by an increased DNA synthesis. This could be linked to the malignant transformation or could indicate a different responsiveness of the normal B cell subset from which B-CLL cells arise.

IL-2 is able to enhance DNA synthesis by cells from most B-CLL patients (17, 22, 23). Moreover, in selected patients the effects of this interleukin can be enhanced by IFN- $\gamma$  (22) and/or IFN- $\alpha$  (18). Thus, monoclonal B cells provide suitable targets to search for synergy between interleukins. We examined the interactions between IL-4 and IL-2. IL-4 profoundly and constantly suppressed the response to IL-2 in all patient responders to this interleukin. This effect was obtained with low concentrations of IL-4 with a 50% inhibitory concentration of 1.5 U/ml. It was not observed with mock preparations of IL-4, and this effect could be demonstrated with a preparation of IL-4 purified to homogeneity. It was not attributable to a delayed DNA synthesis and did not result in terminal differentiation.

These results indicated a negative interaction between IL-4 and IL-2 itself. Indeed they could be obtained in three different experimental conditions: (a) When an optimal response to IL-2 was obtained by costimulation with anti- $\mu$  antibody; (b) when IL-2 was used alone, in these B-CLL cells able to respond to this interleukin in the absence of first signal (17); and (c) when a synergistic response to IL-2 and IFN- $\gamma$  (20) took place. This latter result is important in view of the previously reported interaction between IFN- $\gamma$  and IL-4 (14, 16).

This negative interaction between IL-4 and IL-2 was unexpected in view of the synergy between these interleukins on T cell proliferative response (15). However, it fits with two recently published sets of data. IL-4 inhibits the development of cytotoxic effector cells that occurs in response to IL-2 (24). IL-4 inhibits the growth of bone marrow-derived pre-B cells (12). As discussed earlier, B-CLL lymphocytes might be representative of a minor normal B lymphocyte population exhibiting an unusual responsiveness to IL-4. This is supported by the observation that IL-4, although able to enhance DNA synthesis by normal polyclonal B cells, partially suppresses the IL-2-dependent proliferation of these cells (Defrance, T., et al., manuscript submitted for publication).

Another early effect of IL-4 on normal resting B cells is the upregulation of the expression of CD23 (16, 25, 26). We thus examined whether this effect could take place in B-CLL lymphocytes. To address this question we selected cells from one patient on the basis of the following criteria: (a) These cells expressed low levels of CD23 and were thus potentially susceptible to upregulation; and (b) these cells were responsive to IL-2 in the absence of anti- $\mu$  antibody, allowing the study of the interaction between both interleukins. The incubation with IL-4 induced an upregulation of CD23, showing that IL-4 could positively act on these monoclonal B cells. This contrasted with the profound negative effect of IL-4 on the proliferative response of the same cells to IL-2. The relationships between these two effects of IL-4 are not yet understood. The preincubation of B-CLL cells with IL-4 neither prevents their subsequent responsiveness to IL-2 (provided IL-4 is removed) nor interferes with the upregulation of CD25 expression by IL-2 itself (see Table IV). Further studies are required to analyze the mechanisms of this negative interaction of IL-4 and IL-2 on the same target B cell.

Our results have important implications for the definition of the effects of IL-4 on B cells. Subsets of B lymphocytes may be insensitive to the growth-promoting effect of IL-4. More importantly, IL-4 can counteract the effects of IL-2 on B cell proliferation. As most B-CLL cells respond *in vitro* to IL-2, this antagonism should be taken into account when evaluating the therapeutic perspectives offered by the use of cytokines in B-CLL patients.

### Summary

B cells from patients suffering from B-type chronic lymphocytic leukemia (B-CLL) are susceptible to the effects of several interleukins. Using the cells from 12 different patients we show that IL-4 does not synergize with anti- $\mu$  antibody for the enhancement of DNA synthesis. Moreover IL-4 profoundly (90%) suppresses the response to IL-2 in the 10 patient responders to this interleukin. This suppression occurs whether IL-2 is used alone, in costimulation with anti- $\mu$  antibody, or in synergy with IFN- $\gamma$ . In no instance did IL-4 induce terminal differentiation. This negative effect of IL-4 can take place in monoclonal B-CLL cells where IL-4 enhances the expression of CD23. IL-4 does not interfere with the upregulation of CD25 by IL-2. Thus, IL-4 may display inhibitory effects on the proliferative response of selected B cell populations. The antagonism between IL-4 and IL-2 has important implications for the potential use of cytokines in the management of B-CLL patients.

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