INTERLEUKIN 4 INHIBITS THE PROLIFERATION BUT NOT THE DIFFERENTIATION OF ACTIVATED HUMAN B CELLS IN RESPONSE TO INTERLEUKIN 2

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B lymphocytes proliferate and differentiate in response to various lymphokines (1, 2). Progress has been made recently in the understanding of the mechanisms leading to a coordinated immune response due to the availability of some of these lymphokines in recombinant form. IL-2 (3), IFN-γ and α (4–6), TNF-α/β (7, 8), a low molecular weight B cell growth factor (LMW BCGF)[1] (9) and a not yet molecularly defined high molecular weight BCGF (10, 11) have been shown to induce proliferation of activated normal human B cells. IL-2 (12), IL-5 (13), BSF2/β2-IFN/IL-6 (14–16) have been shown to induce B cell differentiation. It has been recently demonstrated that mouse (17) and human IL-4 (18) have pleiotropic effects. IL-4 induces proliferation of activated B cells (19, 20) and T cells (21, 22) and the expression of the low affinity receptor for IgE ($Fc\varepsilon R_2$/CD23) (23, 24) on resting B cells (25, 26) as well as on certain B cell lines (27). IL-4 was also found to play a central role in the regulation of IgE production in mouse (28) and in human (29), possibly through the release of IgE binding factors (30). IL-4 was also able to induce IgG and IgM production by *Staphylococcus aureus* strain Cowan-activated B cells (SAC) (31). However, little work has yet dealt with the effect of combinations of lymphokines on the various stages of B cell maturation. We have reported that IFN-γ was enhancing the IL-4-induced proliferation of preactivated B cells, whereas it inhibited the IL-4-induced $Fc\varepsilon R_2$/CD23 expression (20, 25), as well as IgE production (29). IFN-γ was also found to enhance IL-2-mediated B cell differentiation (32, 33). Similarly, partially purified LMW BCGF has been shown to act in concert with IL-4 (20) and IL-2 (34, 35) to promote B cell proliferation.

It was the purpose of this study to investigate the effects of combinations of IL-2 and IL-4 on human B cell proliferation and differentiation. The data indicate that the level of maximal response of preactivated B cells for growth and differentiation is higher with IL-2 than with IL-4 and that IL-4 antagonizes the proliferative effect of IL-2 without altering IL-2-induced differentiation.

Materials and Methods

Factors. Purified IFN-γ (γ4A, 10^7 U/mg) and purified IL-2 (3×10^6 U/mg) were purchased from Amgen Biologicals (Thousands Oaks, CA). 1 U of IL-2 is defined as the amount

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[1] *Abbreviations used in this paper:* BCLL, chronic lymphocytic leukemia B cells; LMW BCGF, low molecular weight B cell growth factor; PI, propidium iodide; SAC, Staphylococcus aureus strain Cowan I.

J. Exp. Med. © The Rockefeller University Press · 0022-1007/88/10/1321/17 $2.00 1321
Volume 168 October 1988 1321-1337

of IL-2 that induces 50% of maximal [^{3}H]TdR incorporation by IL-2-dependent T cells in culture. The optimal concentration of rIL-2 as determined in the B cell proliferation assay using 1-d preactivated anti-IgM blasts was found to be within the range of 5 to 10 IU/ml. Highly purified rIL-4 (from L 929 cells; 1.2 × 10^7 U/mg) was a kind gift of Dr. Arai (DNAX, Palo Alto, CA) (36). 1 U of IL-4 is defined as the amount of IL-4 required to cause half-maximal stimulation of tritiated thymidine ([^{3}H]TdR) uptake by 5 × 10^3/200 μl T cells, which were preactivated for 3 d with PHA and then extensively washed. Since preliminary experiments had determined that optimal proliferation of anti-IgM blasts was reached with a concentration of purified rIL-4 of 500 U/ml, all preincubation experiments were performed with 500 U/ml of rIL-4. A commercial preparation of a LMW BCGF partially purified from the culture supernatants of PHA-stimulated PBL was obtained from Cellular Products, Inc. (Buffalo, NY) and will be referred to in the text as LMW BCGF.

Mitogens. Insolubilized rabbit anti-human IgM antibody was purchased from Bio-Rad Laboratories (Richmond, CA). Formalinized SAC was purchased as Pansorbin from Calbiochem-Behring Corp. (La Jolla, CA).

Antibodies. The monoclonal and polyclonal antibodies used for phenotyping the B cell preparations were purchased from the following manufacturers: Becton-Dickinson Monoclonal Center (Mountain View, CA): Leu 3 (CD4), Leu 5 b (CD2), Leu 7, Leu 11 (CD16), Leu 12 (CD19), Leu M3 (CD14); Ortho Pharmaceutical Corp. (Raritan, NJ): OKT3 (CD3), OKT4 (CD4), OKT8 (CD8), OKT11 (CD2); Coulter Immunology (Hialeah, FL): B1 (CD20), MO1 (CD11) and MO2 (CD14); Behring AG (Marburg, Federal Republic of Germany): $F(ab')_2$ fragments anti-human Ig coupled to FITC; Grub (Vienna, Austria): FITC-conjugated $F(ab')_2$ fragments of goat anti-mouse Ig used in the indirect immunofluorescence assays. The neutralizing rabbit anti-IL-4 serum was raised in our laboratory and was prepared as follows. Male rabbits were immunized subcutaneously and intramuscularly with 100 μg of *Escherichia coli*-derived rIL-4 in 1 ml CFA (Difco Laboratories, Detroit, MI). The animals were boosted twice with rIL-4. Blood was collected 3 wk after the third immunization and the antiserum was isolated. The Ig fraction was purified on protein A columns (Bio-Rad Laboratories). This antiserum is specific for IL-4: it blocks IL-4-induced IgG/IgM production from SAC blasts (31), IL-4-induced proliferation of T and B cells, and IL-4-induced CD23 expression on the Burkitt lymphoma cell line Jijoye (Chrétien, I., A. van Kimmenade, M. K. Pearce, J. Banchereau, J. S. Abrams, submitted for publication). The anti-IL-4 antiserum did not block the biological activities of IL-2 either on T cells (proliferation) or on B cells (proliferation and differentiation). The biological activities of rIL-4 originating from different sources (L929 cells, CHO cells, and *E. coli*) were equally blocked by the anti-IL-4 antiserum. A neutralizing anti-IFN-γ serum raised in the laboratory was used as control.

B Cell Preparations. B cells were isolated from tonsils. Mononuclear cells were separated by standard Ficoll/Hypaque gradient method. B cells were obtained by twice rosetting with aminoethylisothiouronium (AET; Sigma Chemical Co., St. Louis, MO)-treated SRBC. The B cell-enriched populations obtained were typically >95% surface Ig-positive, >95% B1 (CD20) antigen-positive. Less than 1% of the cells expressed the T cell markers CD2, CD3, CD4, and CD8, or the monocyte markers CD11 and CD14. Less than 1% of the cells reacted with the NK cell markers Leu 7 and Leu 11. Purified B cells were then further separated according to size using a discontinuous gradient of Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) consisting of four solutions (1.075, 1.070, 1.060, and 1.055 g/ml) sequentially layered on top of each other (3 ml of each solution in a 15-ml centrifuge tube, No. 2095; Falcon Labware, Oxnard, CA). 5 × 10^7 purified B cells were laid on top of the gradient and centrifuged for 20 min at 550 *g* at 20°C. Resting B cells were recovered in the pellet, below the solution of Percoll of the highest density (ρ > 1.075 g/ml).

Culture Conditions and Assays. Purified B cells were cultured in Iscove's medium enriched with 50 μg/ml human transferrin, 0.5% BSA, 5 μg/ml bovine insulin, oleic, linoleic, and palmitic acids (all from Sigma Chemical Co.), and 2% heat-inactivated FCS (Industrie Biologique Française, Genevilliers, France) as described by Yssel et al. (37) for proliferation assays. For differentiation assays, complete RPMI 1640 medium (Flow Laboratories, Irvine, CA) supplemented with selected heat-inactivated FCS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (all from Flow Laboratories), 50 μg/ml gentamicin (Laboratoires UNICET, Levallois-Perret, France), and 2% Hepes was used.

Proliferation Assay. B lymphocytes adjusted at 2×10^6 cells/ml in enriched Iscove's medium described above were stimulated by insolubilized anti-IgM antibody (10 μg/ml) for 24 h. After activation, B cell blasts were harvested and centrifuged over Ficoll/Hypaque gradient to remove nonviable cells and anti-IgM beads. They were then resuspended at 10^6 cells/ml in enriched Iscove's medium and dispensed under a volume of 50 μl into wells of microtiter trays. Factors to be tested for their B cell growth-promoting activity were added to reach a final volume of 100 μl. Cells were pulsed with [^{3}H]TdR (1 μCi/well, sp act 25 Ci/mmol; CEA, Saclay, France) during the last 16 h of the culture period. [^{3}H]TdR uptake was measured by standard liquid scintillation counting techniques after harvesting, using a scintillation counter (LKB Instruments, Bromma, Sweden). The proliferative response of B cell blasts was measured at different intervals from the beginning of culture (from day 2 to day 6). In some experiments, B cell blasts generated after 1 d of preactivation with SAC (0.05%) were also used.

Cell Cycle Analysis. The flow cytometric measurement of total DNA content was done using three different techniques: a single staining method with propidium iodide (PI), a single staining method using Hoechst 33 342 as described by Taylor (38), and a double-staining method combining bromodeoxyuridine (BrdUrd), which is incorporated in place of thymidine in DNA during S phase of the cell cycle, and PI, which stains double-stranded DNA. This technique was performed according to Dolbeare et al. (39), with some modifications as described previously (20). DNA histograms, obtained after PI or Hoechst single staining, were analyzed using the mathematical model of Dean and Jett (40). Each DNA histogram was built after acquisition of 2×10^4 cells.

Differentiation Assay. High density tonsillar B cells ($\rho > 1.075$ g/ml) at 2×10^6 cells/ml in complete RPMI 1640 medium (as described above) were stimulated for 2 d with SAC used at a final concentration of 0.05%. To remove nonviable cells and SAC particles, activated B cells were centrifuged (15 min, 550 *g*) on a gradient consisting of two overlaid solutions of Percoll with densities of 1.077 g/ml and 1.055 g/ml. Viable B cell blasts were recovered at the interface between the two solutions. B cell blasts resuspended in complete RPMI medium were then dispensed (10^5 cells/well) into wells of flat-bottomed microtiter trays (Falcon Labware). Factors to be tested were added in appropriate volumes to reach a final culture volume of 200 μl per well. Each culture point was performed in quadruplicate. Supernatants were harvested 4 d after the onset of the second culture and were kept at −20°C until Ig levels were determined by ELISA. In some experiments, SAC-activated B cell blasts were further separated into CD25$^+$ (Tac$^+$) and CD25$^-$ (Tac$^-$) populations with the FACS after indirect immunofluorescence staining using an anti-CD25 mAb (B1 49.9 mAb; Immunotech, Marseille Luminy, France) and FITC-conjugated F(ab')$_2$ fragments of goat anti-mouse Igs (Grub). The two populations were assayed for proliferation and differentiation using the culture conditions described above.

Assay of Ig Production. The cumulative total Ig in the culture was determined by double sandwich ELISA as described by Voller (41). Briefly, microtiter plates (Immunoplates; Nunc, Roskilde, Denmark) were coated with 200 μl of appropriate dilutions of specific anti-IgG, anti-IgM (Behring AG, Marburg, Federal Republic of Germany) or anti-IgA (Biosys, Compiègne, France) in pH 9.6 carbonate buffer overnight at 4°C. After washing, 200-μl serial dilutions of IgG, IgA, and IgM standards (Behring) in PBS Tween 0.05% were added to the respective plates as calibration curves. Dilutions of culture supernatants were added at the same time. After 2 h at room temperature, the plates were washed, and 200 μl of diluted specific alkaline phosphatase–conjugated anti-IgG, anti-IgM (Behring), or anti-IgA (Biosys) was added. After 2 h at room temperature, plates were washed and 200 μl *p*-nitrophenylphosphate (Sigma Chemical Co.) in diethanolamine buffer was added. Plates were incubated at 37°C, and OD were measured at 405 nm by using an Autoreader (MR 580; Dynatech Laboratories, Inc., Alexandria, VA). OD data were collected and were processed with an Apple IIe computer. Concentrations of Ig in culture supernatants were expressed as nanograms per milliliter.

Preculture Experiments. B cell blasts (generated after 24 h of stimulation with insolubilized anti-IgM or SAC) or high density resting B cells were cultured for 24 h at a cell density of 2×10^6 cells/ml in complete RPMI 1640 medium alone or with rIL-4 (500 U/ml). After preculturing, B cells were centrifuged (15 min, 550 *g*) on a Percoll solution of 1.055 g/ml.

Viable cells recovered in the pellet were then washed and tested for their capacity to proliferate in response to different factors. When resting B cells were used for preculture experiments, the factors to be tested in the second culture period were used in costimulation with insolubilized anti-IgM antibody (at a final concentration of 10 μg/ml). The proliferative response was routinely measured 3 or 4 d after the onset of the second culture with a [^{3}H]TdR pulse during the last 16 h of culture.

Results

rIL-2-dependent B Cell Proliferation of Preactivated B Cells Is Inhibited by rIL-4. We have demonstrated earlier that IL-4-induced B cell proliferation was enhanced by semipurified LMW BCGF and IFN-γ (20). Since IL-2 displays B cell growth-promoting activity and has also been demonstrated to act in cooperation with other B cell growth factors (34, 35), we studied the proliferation of activated B cells in response to combinations of rIL-4 and rIL-2. For this purpose, a two-step assay was performed. Highly purified tonsillar B cells were first preactivated for 24 h with insolubilized anti-IgM antibody (10 μg/ml) or SAC, then recultured for an additional period of 3 d with either purified rIL-4, purified rIL-2, or with a combination of both lymphokines. B cell proliferation was monitored by [^{3}H]TdR uptake. As illustrated in Table I, at optimal concentration of both lymphokines (10 U/ml for rIL-2; 500 U/ml for rIL-4) the levels of [^{3}H]TdR incorporation obtained with rIL-2 were significantly higher than those obtained with rIL-4. Surprisingly, when preactivated B cells were cocultured with rIL-4 and rIL-2, B cell proliferation was lower than that observed with rIL-2 alone and was in most cases comparable to the levels of proliferation obtained with rIL-4 alone (Exps. 1, 2, 4, 5, Table I). In some experiments (Exps. 3 and 6, Table I), addition of rIL-4 to rIL-2-stimulated cultures did not reduce the levels of [^{3}H]TdR uptake to these obtained with rIL-4 alone, although the proliferative response to rIL-2 was significantly inhibited. It is also shown (Table I, Exp. 6) that relatively low amounts of rIL-4 (20 U/ml) can significantly reduce the growth-promoting effect of rIL-2, and that the inhibitory effect of rIL-4 on rIL-2-mediated proliferation is also observed when SAC is used instead of anti-IgM antibody for preactivation of B cells. In some experiments, (one of five, approximately) the proliferation in response to rIL-4 was higher than the proliferation

Table I

rIL-4 Inhibits the rIL-2-mediated Proliferation of Preactivated B Cells

	[^{3}H]TdR incorporation					
	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Exp. 6
	(cpm × 10^{-3} ± SD)					
0	0.6 ± 0.2	2.8 ± 0.4	5.8 ± 0.8	5.6 ± 0.2	18.2 ± 1.8	1.5 ± 0.3
rIL-4	11.6 ± 1.0	13.2 ± 0.2	11.8 ± 0.9	12.9 ± 0.4	54.7 ± 5.4	8.1 ± 0.1
rIL-2	31.5 ± 0.8	24.3 ± 0.2	85.4 ± 4.0	28.3 ± 0.6	98.1 ± 2.4	64.9 ± 1.7
rIL-2 ± rIL-4	13.8 ± 0.9	16.0 ± 0.7	43.9 ± 10.0	17.9 ± 1.8	59.9 ± 3.6	32.9 ± 2.5

Exps. 1-5 were performed on anti-IgM blasts recovered after 1 d of preactivation. Exp. 6 was performed on SAC blasts recovered after 1 d of preactivation. Each value represents the mean ± SD of triplicate determinations. rIL-2 was used at the final concentration of 10 U/ml. rIL-4 was used at the final concentration of 500 U/ml (Exp. 1-4); 100 U/ml (Exp. 5) and 20 U/ml (Exp. 6).

obtained in response to rIL-2, and the proliferative response to the combination of rIL-2 and rIL-4 was equal to that obtained in response to rIL-4 (not shown here). The cell cycle studies performed using the double-staining BrdUrd/PI, or the single-staining systems (PI or Hoechst 33 342) confirmed the [^{3}H]TdR uptake data. B cell blasts generated after 2 d of preactivation with SAC were recultured for 3 d with rIL-2 (10 U/ml), rIL-4 (500 U/ml), or rIL-2 + rIL-4. To prevent cycling cells from returning to G_0/G_1, colcemid (0.1 μg/ml) was added during the last 24 h of the culture. Data displayed in Table II show that rIL-4 significantly reduces the number of cells entering into cycle in response to rIL-2. The inhibition of the rIL-2-mediated B cell proliferation was a function of the concentration of rIL-4 used in the assay (Fig. 1). Kinetics experiments (Fig. 2) demonstrated that the inhibitory effect of rIL-4 was not simply due to a delayed proliferative response to rIL-2, since inhibition could be observed from day 3 up to day 6 after the onset of the culture. When [^{3}H]TdR uptake was measured at earlier time points (12, 24, and 48 h), it was consistently observed that the peak of [^{3}H]TdR uptake in response to rIL-2 was not precipitated in the presence of rIL-4 and that rIL-4 could inhibit the growth promoting effect of rIL-2 as early as 24 h after the onset of the culture (data not shown). To rule out the possibility that the antagonizing activity of IL-4 could be mediated via a non-B cell population, phenotypic analysis were performed on SAC blasts that were recultured for 2 additional d with rIL-2 (10 U/ml), rIL-4 (500 U/ml) or rIL-2 plus rIL-4. As illustrated in Fig. 3, immunofluorescence stainings performed with anti-CD2 and anti-CD20 mAbs established that these three cell populations were composed of >95% B cells, whereas T cells remained under detection levels. Taken together these data indicate that IL-4 can at least partially inhibit the proliferative response of activated B cells to IL-2 and suggest that the antagonizing effect of IL-4 is exerted directly on B cells.

Effect of Preculture with rIL-4 on the Subsequent Proliferative Response of B Cells to rIL-2. To further explore the inhibitory mechanism of IL-4 on IL-2-induced B cell proliferation, experiments were designed in which B cells were first preincubated with rIL-4 for 24 h before culturing with rIL-2. In the first set of experiments, B cell blasts

TABLE II
rIL-4 Reduces the Number of B Cells Entering into Cycle Upon rIL-2 Stimulation

	Cells in S and G_2/M		
Exp.	rIL-4	rIL-2	rIL-4 + rIL-2
	%	%	%
1	33	41	35
2	23	45	24
3	11	24	13
4	19	25	19

B cells blasts generated after 2 d of preactivation with SAC were further recultured for 3 d with rIL-4 (500 U/ml); rIL-2 (10 U/ml); or rIL-4 + rIL-2. Colcemid (0.1 μg/ml) was added during the last 24 h of the culture to block cells in G2/M. In Exp. 1 and 4, DNA staining was performed using BrdUrd and PI. In Exp. 3, DNA staining was performed with Hoechst 33 342. In Exp. 2, DNA staining was performed with PI.

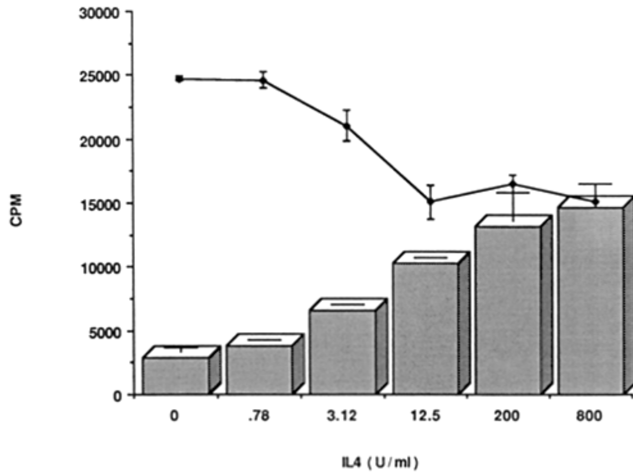

FIGURE 1. The inhibitory effect of rIL-4 is a function of rIL-4 concentration. 1-d preactivated anti-IgM blasts (5×10^4 blasts/well) were recultured for 3 d with purified rIL-2 (10 U/ml) with or without various concentrations of purified rIL-4. The histogram bars represent the proliferative response of anti-IgM blasts to rIL-4 alone, the curve represents the proliferative response of the blasts cocultured with rIL-2 and rIL-4. Representative of three experiments.

generated after 24 h of activation with SAC were further incubated for 24 h with or without rIL-4 (500 U/ml) and then recultured for 72 h with rIL-2. The results of these experiments, illustrated in Fig. 4, indicate that pretreatment of SAC-activated B cells with rIL-4 decreases their subsequent proliferation in response to rIL-2. Similarly, B cell blasts preactivated with anti-IgM, which were incubated with rIL-4 for 24 h, displayed a decreased responsiveness to the growth-promoting effect of rIL-2 compared with control blasts incubated with medium (data not shown). In a second set of experiments, high density tonsillar B cells ($\rho > 1.075$ g/ml) were precultured with medium alone or with rIL-4 (500 U/ml) for 24 h and then assayed for proliferation in response to rIL-2, rIL-4, or rIL-2 plus rIL-4 in costimulation with insolubilized anti-IgM antibody. The data illustrated in Fig. 5 *A* show that pretreatment of resting B cells with rIL-4 does not alter their capacity to proliferate subsequently in response to rIL-2 and anti-IgM antibody. Comparable results were obtained when the period of incubation with rIL-4 was prolonged to 48 h (data not shown). The

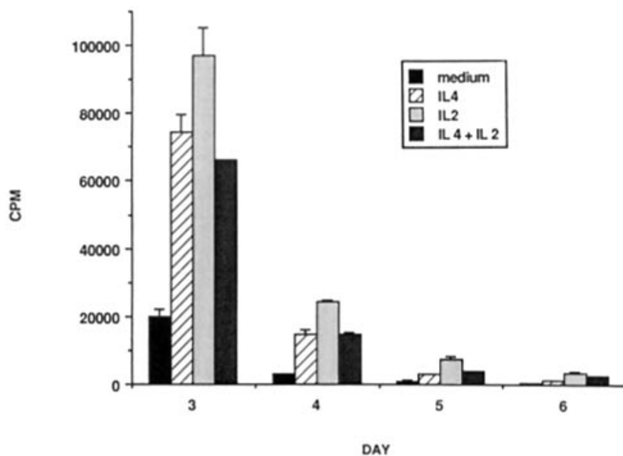

FIGURE 2. rIL-4 does not modify the time kinetics of the proliferative response to rIL-2. 1-d activated anti-IgM blasts were recultured for different lengths of time in the presence of rIL-4 (500 U/ml) (▨), rIL-2 (10 U/ml) (▤), or rIL-4 plus rIL-2 (▦). The [^{3}H]TdR pulse was performed during the last 16 h of the culture period. Representative of three experiments.

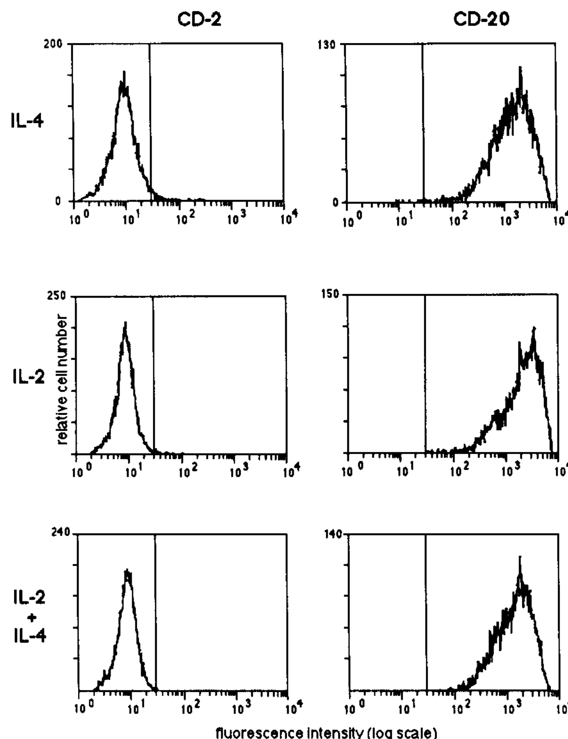

FIGURE 3. Phenotypic analysis of B cell blasts recultured with rIL-2, rIL-4, or rIL-2 plus rIL-4, B cell blasts generated after 48 h of activation with SAC were recultured for 48 h with rIL-4 (500 U/ml), rIL-2 (10 U/ml), or rIL-4 plus rIL-2 and stained with a FITC conjugated pan-T cell marker (anti-CD2 mAb) or a FITC conjugated pan-B cell marker (anti-CD20 mAb). The negative control was performed with a nonrelated mAb of the same isotype. The right limit of the histogram obtained with the negative control is figured by a vertical line. Abscissa fluorescence intensity (log scale). Ordinate relative cell number.

addition of rIL-4 together with rIL-2 and anti-IgM antibody to rIL-4-pretreated cells during the second culture reduces the rIL-2-mediated proliferation to the level of proliferation induced by rIL-4 itself (Fig. 5 *B*). This was observed as well for the control samples that had been pretreated with medium during the first culture period

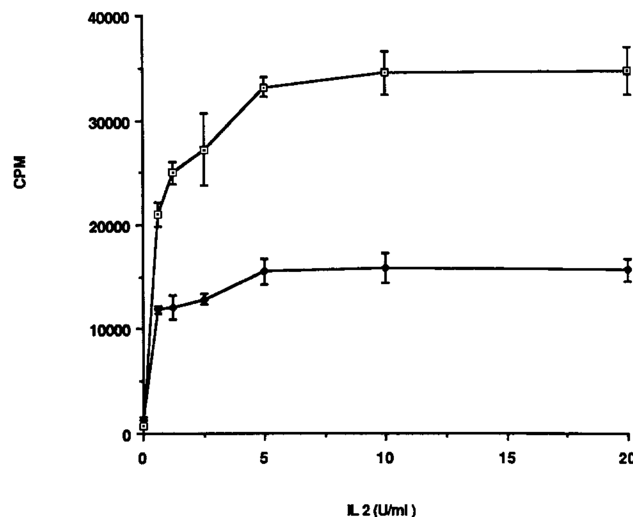

FIGURE 4. Preculture of 1 d preactivated SAC blasts with rIL-4 reduces their capacity to subsequently proliferate in response to rIL-2. B cell blasts generated after 24 h of activation with SAC were submitted to a further incubation with rIL-4 (500 U/ml) (♦) or medium (⊡) during 24 h before the second culture performed in the presence of increasing concentrations of rIL-2. Proliferative response was measured 4 d after the onset of the second culture. With [^{3}H]TdR added 16 h before harvesting. Representative of four experiments.

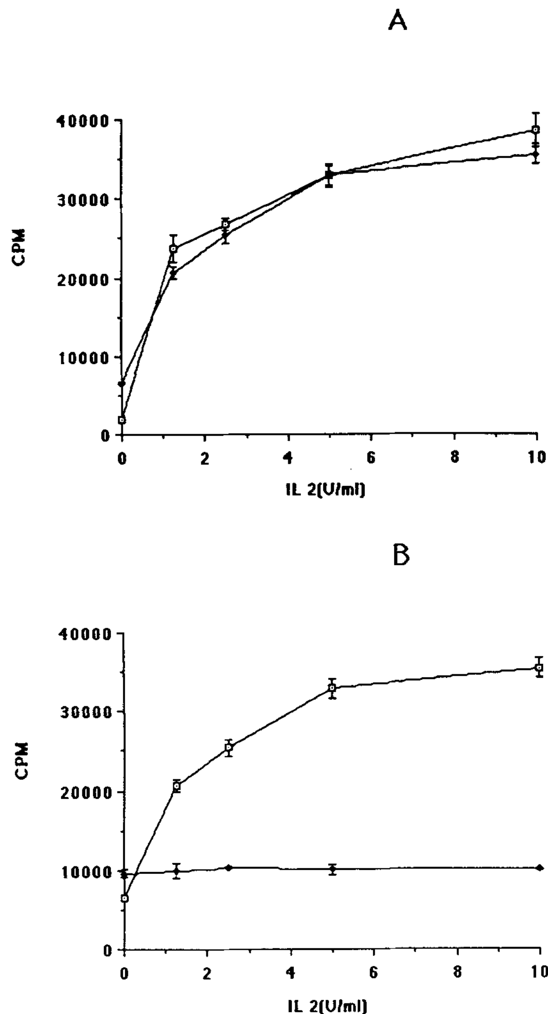

FIGURE 5. Preculture of resting B cells with rIL-4 does not alter their capacity to proliferate in response to rIL-2 plus anti-IgM. Resting B cells recovered in the high density fraction ($\rho > 1.075$ g/ml) after centrifugation on Percoll gradients were first incubated with medium (⊡) or rIL-4 (500 U/ml), (◆) for 24 h. Viable cells were then recovered and assayed for proliferation in the presence of increasing concentrations of rIL-2 and insolubilized anti-IgM antibody (10 μg/ml). (*A*) A comparison of the proliferative responses of the IL-4 pretreated cells and control cultures. (*B*) rIL-4 pretreated cells were further recultured either with rIL-2 alone plus anti-IgM (⊡) or with the combination of rIL-2 plus rIL-4 (500 U/ml) and anti-IgM (◆), all three reagents being added at the onset of the second culture. Proliferative response was measured 4 d after the onset of the second culture, [^{3}H]TdR being added 16 h before harvesting. Representative of four experiments.

(data not shown). These data suggest that IL-4 can affect the capacity of B cells to proliferate in response to IL-2 only when these cells have received an activation signal in vitro.

rIL-4 Does Not Inhibit rIL-2-induced B Cell Differentiation. Since IL-4 can drive activated B cells to differentiate and produce IgG and IgM (31), we next studied whether IL-4 inhibited the IL-2-mediated proliferation of activated B cells by enhancing their differentiation. To test this hypothesis, B cell blasts generated from high density tonsillar B cells after 2 d of activation with SAC were recultured with rIL-4, rIL-2, or the combination of both lymphokines at optimal and suboptimal concentrations. B cell differentiation was monitored by measurement of Ig content (IgG, IgA, and IgM) in the culture supernatants 4 d after the onset of culture. Data illustrated in Table III show that, whatever the concentration of rIL-4 used in combination with rIL-2, no inhibition of rIL-2-mediated IgG production is observed. At optimal con-

TABLE III

rIL-4 Does Not Suppress rIL-2-mediated IgG and IgA Production

rIL-4	IgG				IgA			
	0*	0.3	1.25	10	0*	0.3	1.25	10
U/ml	*ng/ml* × 10^{-2}				*ng/ml* × 10^{-2}			
0	4.6 ± 0.2	17.7 ± 0.3	30.6 ± 4.0	42.0 ± 2.0	1.2 ± 0.4	4.1 ± 0.8	9.0 ± 0.7	10.5 ± 1.0
80	12.9 ± 0.8	32.3 ± 3.0	40.4 ± 0.5	46.0 ± 2.0	2.2 ± 0.08	8.9 ± 0.1	9.2 ± 0.02	12.8 ± 0.9
400	17.5 ± 0.6	30.6 ± 2.0	38.9 ± 4.0	35.9 ± 2.0	1.9 ± 0.6	8.4 ± 0.5	11.5 ± 1.0	13.3 ± 1.0
2,000	21.2 ± 0.9	32.5 ± 2.0	43.7 ± 2.0	46.9 ± 4.0	2.0 ± 0.09	5.4 ± 0.09	10.2 ± 0.6	11.8 ± 2.0

2-d activated SAC blasts were recultured for 4 d with combinations of purified rIL-2 and rIL-4 used at optimal or suboptimal concentrations. Each value represents the mean ± SD of culture quadruplicate. Representative of four experiments.

* rIL-2 (U/ml).

centration of rIL-2 (10 U/ml), rIL-2-induced IgG production is not affected by rIL-4, whereas at suboptimal concentrations of rIL-2 (0.3 and 1.25 U/ml), the effects of rIL-4 and rIL-2 on IgG production are roughly additive. Similar results were obtained with the production of IgM (data not shown). It is noteworthy that rIL-2-mediated IgA production is not suppressed by rIL-4, which by itself induces only a very weak IgA production. To further explore the apparently contradictory effects of IL-4 on IL-2-mediated proliferation and IL-2-mediated differentiation, B cell blasts recovered after 2 d of preactivation with SAC were sorted according to the expression of CD25. $CD25^+$ and $CD25^-$ B cells were then assayed for proliferation and differentiation in response to optimal concentrations of rIL-4, rIL-2, or rIL-2 plus rIL-4. Data illustrated in Table IV demonstrate that the $CD25^+$ population proliferates and differentiates in response to both rIL-2 and rIL-4. In addition, rIL-4 was found to inhibit the proliferation of $CD25^+$ B cells in response to rIL-2 without affecting rIL-2-induced differentiation. The $CD25^-$ B cells did not proliferate nor did they differentiate in response to rIL-4, rIL-2, or to the combination of both lymphokines (data not shown). Taken together, these data demonstrate that IL-4 does not inhibit IL-2-mediated B cell proliferation through an enhancement of B cell differentiation.

The Inhibitory Effect of rIL-4 Is Blocked by a Neutralizing Anti-IL-4 Antiserum. To rule out the possibility that the inhibitory effect of rIL-4 could be mediated by contaminants of the rIL-4 preparations, anti-IgM-activated B cells blasts were recultured for 4 d with rIL-2 (10 U/ml), rIL-4 (20 U/ml), rIL-2 plus rIL-4, or rIL-2 plus rIL-4 in the presence of a 1/500 dilution of a neutralizing rabbit polyclonal anti-IL-4 antiserum (Chrétien, I., et al., submitted for publication). A neutralizing rabbit anti-IFN-γ antiserum was used as control and added at the same dilution to cultures stimulated with rIL-2 and rIL-4. Additionally, to determine the minimum period of contact required for rIL-4 to develop its inhibitory effect, the anti-IL-4 antiserum was added at different time points during the culture (at the onset of the culture, and 7, 16, 24, or 40 h after the onset of the culture). The results illustrated in Fig. 6 represent the proliferative response of 1-d preactivated anti-IgM blasts stimulated with rIL-2 plus rIL-4 in the presence of the anti-IL-4 antiserum or of the control

TABLE IV

Effect of the Combined Action of rIL-2 and rIL-4 on the Proliferation and Differentiation of $CD25^+$ B Cell Blasts

	$[^3H]TdR$ uptake	IgM production
	cpm × 10^{-3}	*ng/ml × 10^{-2}*
Medium	0.5 ± 0.07	1.4 ± 0.08
rIL-2*	59.5 ± 0.1	14.9 ± 1.0
rIL-4‡	9.9 ± 0.6	7.6 ± 0.4
rIL-4 + rIL-2	14.6 ± 0.4	14.4 ± 0.8

B cell blasts recovered after 2 d of activation of high density tonsillar B cells with SAC were positively sorted according to the expression of CD25. Proliferation of $CD25^+$ blasts was determined after 72 h of culture, and IgM production was evaluated after 96 h of culture.

* Purified rIL-2 was used at 10 U/ml.

‡ Purified rIL-4 was used at 500 U/ml.

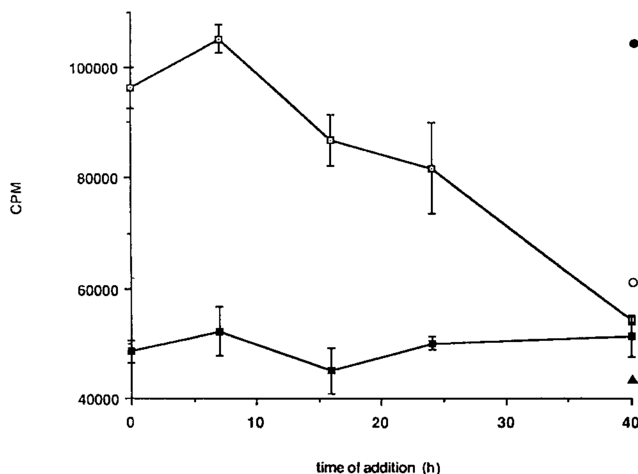

FIGURE 6. Effect of delayed addition of anti-IL-4 neutralizing serum to B cell blasts cocultured with rIL-4 and rIL-2. B cell blasts generated after 1 d of preactivation with insolubilized anti-IgM were recultured with rIL-2 (10 U/ml), rIL-4 (20 U/ml), rIL-2 + rIL-4, rIL-2 + rIL-4 + anti-IL-4 (1/500) or rIL-2 + rIL-4 + control serum (1/500). Anti-IL-4 neutralizing antiserum (⊡) or control antiserum (■) (anti-IFN-γ antiserum) were added at different time points (at the onset of the culture or 7, 16, 24, or 40 h after the onset of the culture). The proliferative response was measured after 4 d of culture, [^{3}H]TdR being added 16 h before harvesting. The [^{3}H]Thd uptake levels in response to rIL-2, rIL-4, rIL-2 plus rIL-4 in the absence of antiserum were, respectively: 106 ± 4.0 × 10^3 cpm (●); 44 ± 4.0 × 10^3 cpm (▲) and 62 ± 2.0 × 10^3 cpm (O). Background [^{3}H]TdR uptake of anti-IgM blasts cultured in medium alone was: 6 ± 0.5 × 10^3 cpm. Representative of three experiments.

antiserum. In the absence of antibody the [^{3}H]TdR uptake in cultures of anti-IgM blasts stimulated with rIL-4, rIL-2, or rIL-2 plus rIL-4 were, respectively: 44 ± 4.0 × 10^3 cpm, 106 ± 4.0 × 10^3 cpm, and 62 ± 2.0 × 10^3 cpm. When anti-IL-4 antiserum is added at the onset of the culture or 7 h after the onset of the culture, the inhibitory effect of rIL-4 is totally blocked (levels of [^{3}H]TdR uptake being respectively, 90 and 98% of the level of [^{3}H]TdR uptake induced by rIL-2 alone). After 24 h of culture, addition of anti-IL-4 antiserum no longer blocks the inhibitory effect of rIL-4. The presence of the control antiserum did not affect rIL-4-mediated inhibition of rIL-2-dependent B cell proliferation, whatever the time of its addition. These data demonstrate that the inhibition observed with rIL-4 is not due to contaminants and that rIL-4 needs to be present for more than 24 h to fully deliver its antagonizing effect.

Discussion

In this report, we have studied the proliferation and differentiation of preactivated tonsillar human B cells in response to combinations of purified rIL-2 and rIL-4. With the majority of the tonsillar B cell preparations, it was found that the maximal proliferative response was higher with rIL-2 than with rIL-4 provided that these factors were both used at optimal concentration. It was repeatedly observed that

combinations of rIL-2 and rIL-4 resulted in proliferation that was lower than that observed with rIL-2 alone and often equivalent to that observed with rIL-4. rIL-4 reduced the levels of [^{3}H]TdR uptake (Table I) induced by rIL-2 as well as the number of cells cycling in response to rIL-2 (Table II). The antagonizing effect of rIL-4 was a function of its concentration, and relatively low amounts of rIL-4 (12.5 U/ml) were sufficient to provide nearly maximum inhibition. Two lines of evidence demonstrated that the inhibitory effects of rIL-4 could not be due to contaminants: first, highly purified rIL-4 was used in all the experiments; second, the inhibitory effect of rIL-4 could be totally reversed by a neutralizing polyclonal anti-IL-4 antiserum. The inhibitory effect of rIL-4 was not due to a delayed response to rIL-2, since the inhibition of [^{3}H]TdR incorporation was observed from day 3 to 6. As shown by phenotypic analysis with anti-CD2 and anti-CD20 mAbs, the cell populations cultured with rIL-2, rIL-4, or rIL-4 plus rIL-2 were composed of >95% B cells, whereas T cells and monocytes remained below the levels of detection. Therefore, it is unlikely that the growth inhibitory effect of IL-4 could be mediated via a non-B cell population.

Treatment of anti-IgM as well as SAC preactivated B cells with rIL-4 for 24 h significantly reduced their subsequent proliferation in response to rIL-2. Delayed addition of the neutralizing anti-IL-4 antiserum to anti-IgM blasts cultured with rIL-4 and rIL-2 established that the inhibitory effect of rIL-4 on rIL-2-induced proliferation required cells to be in contact with rIL-4 for at least 24 h. This also corresponds to the minimum length of time necessary for rIL-4 to display B cell growth factor activity (20). In contrast, resting B cells pretreated with rIL-4 were still able to fully respond to the growth-promoting activity of rIL-2 in combination with anti-IgM antibody. Nevertheless, readdition of rIL-4 together with rIL-2 and anti-IgM antibody in the second culture period could still block the rIL-2-mediated proliferative response of these cells. These findings suggest that in vitro activation of B cells with anti-IgM antibody or SAC before treatment with rIL-4 or together with rIL-4 is necessary for development of the inhibitory effect of rIL-4.

Experiments performed on SAC blasts demonstrated that rIL-4 did not suppress rIL-2-induced Ig synthesis. rIL-4 did not significantly potentiate Ig production induced by rIL-2 (used at optimal concentration), therefore suggesting that the antagonistic activity of rIL-4 on B cell proliferation induced by IL-2 was not due to an enhancing effect of rIL-4 on rIL-2-mediated differentiation. However, the possibility remained that the apparently unchanged levels of Ig production detected in the rIL-2 plus rIL-4-stimulated cultures compared with the rIL-2-stimulated cultures was in fact reflecting the sum of rIL-4-induced Ig secretion and reduced rIL-2-mediated Ig secretion. This hypothesis was ruled out by the IgA results (Table III), which demonstrate that rIL-4 (which does not induce significant production of IgA by itself) does not suppress rIL-2-mediated IgA production.

In view of the fact that IL-4 can cooperate with IFN-γ and LMW BCGF for B cell proliferation (20) and with IL-2 for T cell proliferation (22), the counteracting effect of IL-4 on the B cell growth-promoting activity of IL-2 was an unexpected finding. However, there is now growing evidence that IL-4 can deliver negative signals to various cell types. Indeed, it has been demonstrated that IL-4 suppresses the generation of LAK cells from PBL by IL-2 (42, 43). Moreover, IL-4 has been shown to inhibit the factor-dependent growth of pre-B cells from bone marrow precursors

(44). In addition, our finding that IL-4 antagonizes IL-2-dependent growth of activated normal B cells is in line with the recent data obtained with chronic lymphocytic leukemia B cells (B-CLL), whose IL-2-dependent proliferation is totally blocked by IL-4 (45). Our data thus indicate that the IL-4-mediated inhibition of IL-2-dependent growth of B-CLL cells is not merely due to the transformed state of these cells. This suggests that B-CLL cells might represent the malignant counterpart of the normal B cell subset sensitive to the antagonizing activity of IL-4.

The contrasting effects of IL-4 on IL-2-mediated proliferation and differentiation of preactivated human B cells suggest that these two IL-2-dependent events may be dissociated. Whether this dissociation is linked to the existence of two different B cell subsets, one differentiating and one proliferating in response to IL-2, remains to be determined. However, in line with this hypothesis, there are now reports that tend to support the notion that activated B cells can be separated according to their capacity to proliferate or differentiate in response to T cell factors. It has been shown that the B cell subset that proliferates in response to SAC is different from that which differentiates in response to SAC and irradiated T cells, and that these two populations can be separated by their ability or inability to form rosettes with mouse erythrocytes (46). Moreover, Nakagawa et al. (47) recently demonstrated that SAC-activated B cell blasts can be divided into subsets that either differentiate or proliferate in response to IL-2. It is thus tempting to speculate that the subpopulation proliferating in response to IL-2 is the target of the IL-4 inhibitory effects, whereas the subpopulation differentiating in response to IL-2 is not affected by IL-4. It is likely that the B cell subpopulations differentiating in response to IL-2 and IL-4 may largely overlap, since the differentiative activities of both lymphokines used at optimal concentration are not additive, whereas they are roughly additive at suboptimal concentrations. In a first attempt to separate IL-2- and IL-4-responsive populations, we sorted out $CD25^+$ and $CD25^-$ B cells from a total population of SAC-activated blasts. It was found that B cells that did not express CD25 could not proliferate or differentiate in response to rIL-2 or rIL-4. Thus, cells proliferating and differentiating in response to rIL-2 or rIL-4 were included within the $CD25^+$ subset. These experiments also confirmed, at the level of a B cell subpopulation selected on the expression of the IL-2 receptor, that rIL-4 can antagonize rIL-2-mediated B cell growth without affecting rIL-2-induced Ig production. Presently, we can only speculate on the nature of the mechanism involved in the inhibitory activity of IL-4. An IL-4-mediated downregulation of the expression of the IL-2 receptor heterodimer could be a possible explanation. Binding experiments are presently being performed to challenge this hypothesis.

The data presented in this report demonstrate that, in addition to its stimulatory effects on the immune response, IL-4 delivers inhibitory signals to B cells. This finding adds further complexity to the understanding of the sequence of events that affect a B cell once it has encountered its specific antigen.

Summary

The combined effect of IL-4 and IL-2 on proliferation of anti-IgM antibody or Staphylococcus aureus strain Cowan I (SAC)-preactivated B cells was investigated. It was observed that in most cases, rIL-2 used at optimal concentration induced higher levels of tritiated thymidine ([^{3}H]TdR) uptake than rIL-4 used at optimal concen-

tration. When rIL-4 and rIL-2 were added together, it was repeatedly found that B cell proliferation induced by rIL-2 was significantly reduced and was, in most cases, comparable with the proliferation induced by rIL-4 alone. Cell cycle studies demonstrated that rIL-4 significantly reduced the number of cells entering S and G_2/M phases of the cell cycle upon rIL-2 stimulation. B cell blasts preincubated for 24 or 48 h with rIL-4 displayed a reduced proliferation in response to rIL-2. In contrast, preculture of resting B cells with rIL-4 did not impair their subsequent proliferation in response to rIL-2 plus insolubilized anti-IgM antibody. This suggests that rIL-4 can only exert its inhibitory effect once B cells have received an activation signal. The differentiative activity of rIL-2 measured on B cell blasts preactivated for 2 d with SAC was not altered by rIL-4, which suggests that rIL-4 did not exert its inhibitory activity on rIL-2-induced B cell proliferation by enhancing rIL-2-mediated differentiation. Delayed addition of a neutralizing anti-IL-4 antiserum demonstrated that a period of contact of at least 24 h between IL-4 and B cell blasts was necessary for the development of the antagonistic effect of IL-4 on IL-2-mediated growth of activated B cells. These data demonstrate that IL-4 antagonizes the B cell growth-promoting effect of IL-2 without affecting the differentiation of preactivated B cells in response to IL-2.

We thank Dr. Allan Waitz and Francine Brière for careful review of the manuscript, Isabelle Durand for expert assistance with flow cytometry, Isabelle Chrétien for providing the anti-IL-4 antiserum, and Nicole Courbière for excellent secretarial assistance.

Received for publication 28 March 1988 and in revised form 5 July 1988.

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