

INTERLEUKIN 4 INDUCES SELECTIVE PRODUCTION  
OF INTERLEUKIN 6 FROM NORMAL  
HUMAN B LYMPHOCYTES

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The growth and differentiation of B lymphocytes are regulated by complex mechanisms that involve the action of cytokines such as IL-1, IL-2, IL-4, IL-5, TNF- $\alpha$  and - $\beta$ , IFN- $\gamma$ , - $\alpha$ , and - $\beta$ , low molecular weight B cell growth factor (LMW BCGF), and high molecular weight BCGF (1, 2). In addition, IL-6/IFN- $\beta$ 2/BSF-2 is a potent inducer of Ig secretion in human B cells, and it also has growth factor activity for hybridomas and plasmacytomas (1, 3). Recent evidence suggests that activated normal B cells are capable of secreting cytokines that are involved in the regulation of growth and differentiation of B lymphoid cells, such as IL-1, TNF- $\alpha$ , and IL-6 (4-6). In this report we have examined the production of TNF- $\alpha$ / $\beta$  and IL-6 by extensively purified human B lymphocytes activated by different growth stimuli. The combination of phorbol esters and the calcium ionophore ionomycin was particularly potent in inducing the production of these cytokines. Notably, IL-4 induced significant production of IL-6 in a dose-dependent fashion when added to resting B cells. However, in contrast to several other stimuli, IL-4 did not induce TNF- $\alpha$  or TNF- $\beta$  production, implying a selective action of IL-4 on IL-6 induction.

Materials and Methods

*Reagents.* F(ab')<sub>2</sub> fragments of rabbit polyclonal antibodies to human  $\mu$  H chain (anti- $\mu$ ; Dako Corp., Copenhagen, Denmark) were prepared by pepsin treatment (7) and used at 75  $\mu$ g/ml. The calcium ionophore ionomycin was purchased from Calbiochem-Behring Corp. (La Jolla, CA) and used at a final concentration of 0.5  $\mu$ M. The phorbol esters tetradecanoylphorbolacetate (TPA; Consolidated Midland Corp., Brewster, NY) and phorbol dibutyrate (PDB; Sigma Chemical Co., Poole Dorset, UK) were both used at final concentrations of 10<sup>-8</sup> M. Human rIL-1- $\beta$  (kindly provided by Dr. A. Shaw, Glaxo Institute for Molecular Biology, Geneva, Switzerland) had a specific activity of 5  $\times$  10<sup>7</sup> U/ml. Human rIL-2 was obtained from Cetus Corp. (Emeryville, CA). Human rIL-4 was purchased from Genzyme (Boston, MA), and contained <5 ng LPS per ml IL-4 (i.e., per 20,000 U IL-4). As a source of human IL-5, a supernatant from COS cells transfected with the gene for human IL-5 was used (kindly provided by Dr. T. Honjo, Kyoto, Japan). Human rIL-6 had a specific activity of 10<sup>9</sup> U/ml as determined in the B9 assay (8) and was kindly provided by Dr. L. Aarden, University of Amsterdam, The Netherlands. rIFN- $\gamma$  was a product from Genentech Inc. (South

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San Francisco, CA) kindly provided by G. R. Adolf at Ernst-Boehringer Institute for Arzenimittelforschung (Vienna, Austria) with a specific activity of  $1.2 \times 10^7$  U/mg (bioassay, inhibition of CPE and EMC virus on human carcinoma cells, A549). Human rTNF- $\alpha$  and human rTNF- $\beta$  (9, 10) were kindly provided from Genentech Inc., and had a specific activity of  $7.6 \times 10^7$  and  $10^8$  U/mg protein for rTNF- $\alpha$  and rTNF- $\beta$ , respectively.

**Cell Purification and Culture.** Highly enriched resting human B lymphocytes were isolated from peripheral blood buffycoats by a method developed in our laboratory (Funderud, S., et al., manuscript submitted for publication). Briefly, Dynabeads M450 (Dyna, Oslo, Norway) were coated with the anti-CD19 mAb AB-1, and added to buffycoats at a ratio of five beads per B cell, assuming  $5 \times 10^7$  B cells per buffycoat. The mixture was incubated on a rocking platform for 30 min at 4°C, and cells forming rosettes with AB-1 beads were trapped by a samarium cobalt magnet (Dyna). Nonrosetting cells were subsequently removed by suction. Rosetted cells were washed six times to remove nonrosetting cells. AB-1 beads will detach from the B cells by overnight culture in RPMI 1640 (Gibco Laboratories, Paisley, Scotland) supplemented with 1% FCS. This procedure reads <0.5% T cells, 0.5% monocytes, and 0.1% NK cells, as judged by indirect immunofluorescence staining (Funderud et al., manuscript submitted for publication). In addition, the cells were subjected to two subsequent rounds of purification. First, the highly enriched B cells ( $10^6$ /ml) were exposed to Dynabeads M450 (Dyna) coated with antibodies against CD2 (Dakopatts, Copenhagen, Denmark), CD4 (Dynabeads M450; no. 11106), CD8 (5C2; a kind gift of G. Gaudernack, Oslo, Norway), and the panmonocyte antibody 1D5 (kindly provided by G. Gaudernack). Finally, the cells were incubated with OKM-1 (Ortho Diagnostic Systems Inc., Westwood, MA), OKT3 (Ortho Diagnostic Systems Inc.), OKT11 (Ortho Diagnostic Systems Inc.), anti-Leu-M1 (Becton Dickinson & Co., Sunnyvale, CA), and anti-NKH1 (Coulter Immunology, Hialeah, FL) at saturating conditions, followed by complement-dependent lysis using rabbit complement. The extensively purified B cells contained <0.1% T cells, <0.1% monocytes, and <0.1% NK cells as determined by indirect immunofluorescence or immunoenzymatic stainings of cytospin preparations.

**Detection of TNF and IL-6 in Supernatants of Highly Purified B Cells.** Purified B cells were grown in RPMI 1640 (Gibco Laboratories) supplemented with penicillin, streptomycin and 1% FCS at a concentration of  $10^6$  cells/ml. Supernatants were collected at various time points and assayed for cytokine levels in bioassays described below. TNF levels were determined by the WEHI 164 clone 13 bioassay, which is capable of detecting as low as 0.1 pg/ml rTNF- $\alpha$  (11). To measure TNF- $\beta$  activity, all TNF- $\alpha$  activity was first neutralized by adding 10  $\mu$ g/ml of a neutralizing mAb against TNF- $\alpha$  for at least 30 min before bioassay. 200 ng of this mAb (6H11) neutralizes at least 10 ng of rTNF- $\alpha$  (T. Espevik, unpublished results). Neutralization of TNF- $\beta$  activity in the test supernatants was done by adding rabbit polyclonal antibodies against TNF- $\beta$  ( $2.9 \times 10^7$  neutralizing U/ml; M. R. Shalaby, unpublished results).

Production of IL-6 was determined in a bioassay using the murine hybridoma cell line B11.29 clone B9, which is dependent on IL-6 for growth (8, 12). rIL-1, rIL-2, rIL-4, and rIL-5 had no detectable effects in the cytokine assays used.

## Results

**Effect of IL-4 on IL-6 Production from Purified Human B Cells.** It is well established that IL-4, when given to resting human B cells, promotes a marked increase in CD23 expression (2, 13), although it does not induce a regular G<sub>0</sub>-G<sub>1</sub> entry. It was therefore of interest to investigate the effects of IL-4 on cytokine production in human B cells. As can be seen in Fig. 1, treatment of purified human peripheral blood B lymphocytes with rIL-4 resulted in a marked, dose-dependent secretion of IL-6. Even a dose as low as 1 U/ml of IL-4 was sufficient to induce IL-6 production from purified B cells. It is unlikely that this effect is due to the low amount of contaminating LPS in the rIL-4 preparation, as LPS did not have any effects on IL-6 production in purified B cells over a wide dose range (data not shown). In another experiment, the kinetics of IL-6 production from B cells stimulated with 50 U/ml IL-4 was examined. The

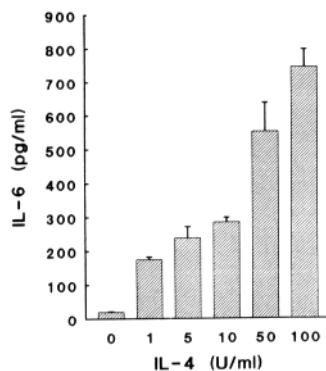


FIGURE 1. IL-4 induction of IL-6 secretion in human B cells. Extensively purified resting B cells were stimulated with different concentrations of IL-4 for 48 h. The supernatants were assayed for IL-6 activity as described in Materials and Methods. Data represent mean (SD) of triplicate measurements from a representative experiment.

results in Fig. 2 show that IL-6 was detected in the supernatants already after 1–2 h, reaching near maximal levels after 48 h of stimulation. The addition of rabbit anti-human rIL-6 serum (W. Fiers, State University of Ghent, Belgium) at a 1:120 dilution completely abolished the IL-6 activity in the test supernatants (data not shown). No TNF activity was detected after IL-4 stimulation (Table I), indicating that IL-4 is a specific inducer of IL-6 production in isolated B cells.

*Effect of Cytokines and Phorbol Esters on IL-6 and TNF Production from Purified B Cells.* Both IL-1 and TNF- $\alpha$  have been shown to induce IL-6 secretion in other cell systems (3, 12). It was therefore of interest to explore the effects of cytokines on TNF and IL-6 production in human B cells. However, neither rIL-1- $\beta$ , rIL-2, rIL-5, rIL-6, rIFN- $\gamma$ , rTNF- $\alpha$ , nor rTNF- $\beta$  induced IL-6 or TNF production in B cells (Table I). Phorbol esters like TPA and PDB activate resting B cells into G<sub>1</sub> (14) and synergize with anti- $\mu$  or the calcium ionophore ionomycin for B cell proliferation. Both TPA and PDB induced a markedly increased production of IL-6, as well as TNF, especially when combined with ionomycin or anti- $\mu$  (Table I). Maximal TNF levels were reached between 14 and 24 h after stimulation, while IL-6 induction tended to be more delayed with maximal levels most often found at 48 h (data not shown).

As treatment of resting B cells with polyclonal anti- $\mu$  activates the cells into the G<sub>1</sub> phase of the cell cycle without marked S phase entry in the absence of cofactors (7), we wanted to test the effects of anti- $\mu$  on cytokine production. As can be seen in Table I, polyclonal anti- $\mu$  alone induced only a modest increase in TNF and IL-6 secretion. However, anti- $\mu$  synergized with phorbol esters (Table I) and IL-4 (Fig.

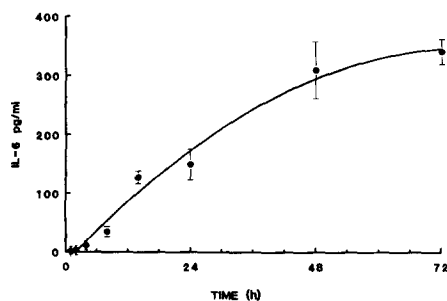


FIGURE 2. Kinetics of IL-4 induced IL-6 secretion in human B cells. B cells were cultured for different time periods in the presence of IL-4 (50 U/ml). IL-6 activity was determined as described in Materials and Methods.

TABLE I  
Production of TNF and IL-6 by Activated Human B Cells

	TNF		IL-6	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
	pg/ml		pg/ml	
Control (medium)	0.8 (0.05)	1.6 (0.1)	10.8 (1.7)	20.0 (1.1)
Rabbit anti- $\mu$	4.2 (0.9)	2.7 (0.7)	104.0 (3)	58.2 (8.3)
TPA	12.3 (0.8)	6.7 (0.6)	51.4 (9.0)	243.0 (29)
Ra $\mu$ + TPA	52.0 (0.8)	16.3 (1.0)	614.0 (56)	2,350.0 (444)
PDB	14.5 (1.5)	6.6 (0.5)	128.0 (15)	172.0 (12)
Ionomycin	<0.4	0.6 (0.1)	14.1 (1.6)	22.0 (3.4)
Ionomycin + TPA	ND	28.1 (3.8)	ND	1,177.0 (95)
Ionomycin + PDB	70.0 (3.0)	86.0 (13.5)	1,357.0 (55)	>3,900.0
IL-1 (10 ng/ml)	<0.4	1.8 (0.1)	16.0 (3.4)	28.7 (2.3)
IL-1 (50 ng/ml)	ND	2.0 (0.2)	ND	8.0 (0.6)
IL-2 (25 U/ml)	<0.4	ND	10.0 (0.4)	ND
IL-4 (50 U/ml)	<0.4	3.4 (0.1)	227.0 (8.0)	550.0 (86)
IL-5 (1/1,000)	0.7 (0.2)	ND	<10	ND
IL-6 (10 U/ml)	1.1 (0.2)	1.3 (0.1)	12.4	30.0 (2.0)
IL-6 (20 U/ml)	ND	1.6 (0.1)	ND	13.0 (0.4)
IL-6 (50 U/ml)	ND	1.6 (0.2)	ND	36.0 (0.1)
IFN- $\gamma$ ( $10^3$ U/ml)	0.9 (0.02)	ND	14.0 (2.0)	ND
TNF- $\alpha$ ( $10^3$ U/ml)			ND	32.5 (1.4)
TNF- $\alpha$ ( $10^4$ U/ml)			<10	19.9 (4.5)
TNF- $\beta$ ( $10^3$ U/ml)			ND	23.3 (3.0)
TNF- $\beta$ ( $10^4$ U/ml)			ND	22.9 (2.1)

Highly purified B cells were incubated with the indicated stimuli for 14 h (TNF) or 48 h (IL-6), and the supernatants were assayed for cytokine activity as described in Materials and Methods. The data represent mean (SD) of triplicate measurements from two representative experiments.

3) for cytokine production. The combination of anti- $\mu$  and IL-4 was almost as efficient in inducing IL-6 production as the combination of ionomycin and PDB (data not shown).

Activated T lymphocytes have been shown to produce both TNF- $\alpha$  as well as TNF- $\beta$  (15). We therefore investigated the relative production of these two cytokines after stimulation of purified B cells with PDB and ionomycin, which represented the stimulus

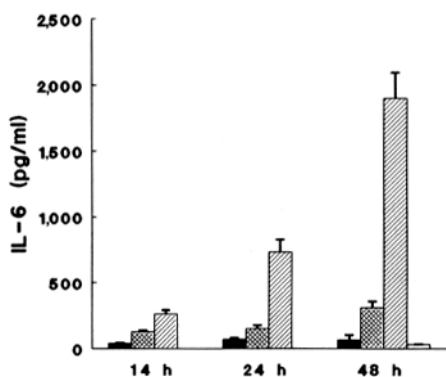


FIGURE 3. Synergistic action of IL-4 and anti- $\mu$  on IL-6 production. Resting B cells were cultured with anti- $\mu$  (■), IL-4 (50 U/ml; ▨), anti- $\mu$  plus IL-4 (▩), or in medium alone (□) for the indicated time periods. IL-6 activity was determined as described in Materials and Methods.

that induced the highest levels of total TNF production (Table I). In different experiments, between 44 and 80% of the TNF activity detected after 48 h of culture was due to presence of TNF- $\alpha$  (data not shown). Moreover, our results indicate that TNF- $\alpha$  production precedes TNF- $\beta$  production in activated B cells (data not shown).

### Discussion

In this report we have examined the production of TNF and IL-6 in peripheral blood B cells. As several cell types present in peripheral blood are capable of secreting these cytokines, it was important to use extensively purified B cells in order to minimize possible effects of contaminating cells. Before use, the cells had therefore been subjected to several rounds of purification by using positive and negative selection with immunomagnetic beads followed by complement-mediated lysis. The results demonstrate that rIL-4 induced a marked secretion of IL-6 in resting B cells, and that anti- $\mu$  significantly potentiated the IL-4-induced IL-6 production. In contrast to findings in other cell systems, such as fibroblasts and endothelial cells (3, 12), rIL-1 and rTNF- $\alpha$  did not induce any significant IL-6 secretion. The selectivity of the IL-4 effect on IL-6 production is further strengthened by the observation that IL-4 did not induce the B cells to produce TNF. None of the other cytokines tested were able to induce IL-6 production when given to resting B cells. This accords well with data by DeFrance et al. (13), who found that of several factors tested, only IL-4 induced increased CD23 expression in human B cells.

Our results also confirm the recent observations that activated human B cells produce TNF- $\alpha$  and TNF- $\beta$  (5), as well as IL-6 (6). Phorbol esters, in particular, seem to be very potent in inducing cytokine secretion in human B cells, especially when combined with ionomycin. However, it is clear that the observed secretion of TNF and IL-6 is not a mere result of cellular activation, as polyclonal anti- $\mu$ , which activates resting B cells into the G<sub>1</sub> phase of the cell cycle (7), only induced a modestly increased secretion of these cytokines when given alone.

TNF- $\alpha$ , TNF- $\beta$ , and IL-6 have all been shown to influence functional responses of B cells (1-3). Available data therefore suggest that B cells may produce and respond to important cytokines, adding to the complexity of the regulation of B cell responses. Furthermore, our finding that IL-4 induces IL-6 production raises the possibility that the induction of IgG secretion in human B cells by IL-4, at least partly, may be due to an autocrine production of IL-6. This emphasizes the difficulties in assessing the effects of a single cytokine given the extensive interaction of cytokines in complex networks.

### Summary

In this paper we have shown that extensively purified human B lymphocytes respond to IL-4 treatment with a marked production of IL-6. Addition of anti- $\mu$  potentiated the effect of IL-4 on IL-6 production. Other cytokines tested like TNF- $\alpha$  and - $\beta$ , IFN- $\gamma$ , IL-1, IL-2, and IL-5 did not induce IL-6 secretion when given to resting B cells. Although B cells generally also produced TNF- $\alpha$  and TNF- $\beta$  upon stimulation, IL-4 did not induce TNF secretion and seemingly had a specific effect on IL-6 production.

*Received for publication 8 May 1989 and in revised form 19 July 1989.*

## References

1. Kishimoto, T. 1985. Factors affecting B cell growth and differentiation. *Annu. Rev. Immunol.* 3:135.
2. O'Garra, A., S. Umland, T. DeFrance, and J. Christiansen. 1988. "B-cell factors" are pleiotropic. *Immunol. Today.* 9:45.
3. Wong, G. G., and S. C. Clark. 1988. Multiple actions of interleukin 6 within a cytokine network. *Immunol. Today.* 9:137.
4. Pistoia, V., F. Cozzolino, A. Rubartelli, M. Torcia, S. Roncella, and M. Ferrarini. 1986. In vitro production of interleukin 1 by normal and malignant human B lymphocytes. *J. Immunol.* 136:1688.
5. Sung, S.-S. J., L. K. L. Jung, J. A. Walters, W. Chen, C. Y. Wang, and S. M. Fu. 1988. Production of tumor necrosis factor/cachectin by human B cell lines and tonsillar B cells. *J. Exp. Med.* 168:1539.
6. Horii, Y., A. Muraguchi, S. Suematsu, T. Matsuda, K. Yoshizaki, T. Hirano, and T. Kishimoto. 1988. Regulation of BSF-2/IL-6 production by human mononuclear cells. Macrophage-dependent synthesis of BSF-2/IL-6 by T cells. *J. Immunol.* 141:1529.
7. Smeland, E., T. Godal, E. Ruud, K. Beiske, S. Funderud, E. A. Clark, S. Pfeifer-Ohlsson, and R. Ohlsson. 1985. The specific induction of *myc* protooncogene expression in normal human B cells is not a sufficient event for acquisition of competence to proliferate. *Proc. Natl. Acad. Sci. USA.* 82:6255.
8. Aarden, L. A., E. R. de Groot, O. L. Shaap, and P. M. Landsdorp. 1987. Production of hybridoma growth factor by human monocytes. *Eur. J. Immunol.* 7:1411.
9. Pennica, D., G. E. Nedwin, J. S. Hayflick, P. H. Seeburg, R. Derynck, M. A. Palladino, W. J. Kohr, B. B. Aggarwal, and D. V. Goeddel. 1984. Human tumor necrosis factor: cDNA cloning, expression, and homology to lymphotoxin. *Nature (Lond.)* 312:724.
10. Gray, P. W., B. B. Aggarwal, C. V. Benton, T. S. Bringman, W. J. Henzel, J. A. Jarrett, D. W. Leung, B. Moffat, P. Ng, L. P. Svedersky, M. A. Palladino, and G. E. Nedwin. 1984. Cloning and expression of cDNA for human lymphotoxin, a lymphokine with tumor necrosis activity. *Nature (Lond.)* 312:721.
11. Espevik, T., and J. Nissen-Meyer. 1986. A highly sensitive cell line, WEHI 164 clone 13, for measuring cytotoxic factor/tumor necrosis factor from human monocytes. *J. Immunol. Methods.* 95:99.
12. Shalaby, M. R., A. Waage, and T. Espevik. 1989. Cytokine regulation of interleukin 6 production by human endothelial cells. *Cell. Immunol.* 121:372.
13. DeFrance, T., J.-P. Aubry, F. Rousset, B. Vanbervliet, J. Y. Bonnefoy, N. Arai, Y. Takebe, T. Yokota, F. Lee, K. Arai, J. de Vries, and J. Banchereau. 1987. Human recombinant interleukin 4 induced Fcε receptors (CD23) on normal human B lymphocytes. *J. Exp. Med.* 165:1459.
14. Gordon, J., M. J. Millsum, G. R. Guy, and J. A. Ledbetter. 1987. Synergistic interaction between interleukin 4 and anti-Bp50 (CDw40) revealed in a novel B cell restimulation assay. *Eur. J. Immunol.* 17:1535.
15. Steffen, M., O. G. Ottman, and M. A. S. Moore. 1988. Simultaneous production of tumor necrosis factor-α and lymphotoxin by normal T cells after induction with IL-2 and anti-T3. *J. Immunol.* 140:2621.