Cytokine-induced Proliferation and Immunoglobulin Production of Human B Lymphocytes Triggered through Their CD40 Antigen

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Summary

Human resting B lymphocytes enter a state of sustained proliferation when incubated with both mouse fibroblastic L cells stably expressing FcγRII/CDw32 and anti-CD40 antibodies. We have explored the effects of 11 recombinant human cytokines (CKs) on induced cell proliferation and immunoglobulin (Ig) production. Interleukin 4 (IL-4) was the only CK able to enhance anti-CD40-induced B cell multiplication as measured by enumeration of viable cells, and interferon γ (IFN-γ) further stimulated this induced proliferation. IL-4 enhanced the production of IgM and IgG by B cells and induced them to produce IgE. Combinations of IL-4 and IL-2 resulted in the production of large amounts of IgM and IgA. Interestingly, IFN-γ did not inhibit the production of IgE by cells stimulated with anti-CD40 and IL-4. None of the tested CK combinations resulted in the production of large quantities of IgG. Therefore, this new culture system represents a unique model to study isotype regulation in highly purified human B lymphocytes, in addition to allowing the generation of long-term factor-dependent human B cell lines.

The maturation of resting B lymphocytes into Ig-secreting cells is a highly regulated phenomenon, thought to be coupled to considerable proliferation, which requires the participation of antigen (Ag),[1] T cells, and probably accessory cells (3) acting through cell-cell interactions via specific membrane Ags and through the release of cytokines (CKs). An approach to understanding the phenomena involved in B cell activation has been made possible through the availability of polyclonal activators including antibodies to surface Ags (4) and recombinant CKs (3). These studies have shown that antibodies against the B cell Ag receptors (sIg) as well as other non-Ig molecules can deliver activation signals to B cells. In particular, the CD40 molecule has recently emerged as a functionally important B cell surface Ag (5, 6). The CD40 Ag is a 277–amino acid glycoprotein whose 172–amino acid extracellular domain has homology with the nerve growth factor receptor (7) and the 55- and 80-kD TNF receptors (8). Soluble anti-CD40 strongly enhances [³H]TdR uptake in costimulated B cells and activates resting B cells as determined by cell size increase (5, 6). More recently, we have shown that anti-CD40 antibodies presented on irradiated transfected L cells stably expressing FcγRII/CDw32 induce a strong and long-lasting proliferation of human B lymphocytes, which allowed us to generate factor-dependent human B cell lines free of EBV infection (9).

The present work is aimed at studying the effects of many different recombinant CKs on the proliferation and Ig production of human B cells activated with anti-CD40. We show that IL-4 is the sole CK able to boost the anti-CD40-induced expansion of B lymphocytes and that IFN-γ enhances this effect. Furthermore, IL-4 induces anti-CD40-activated B cells to produce IgE, and combinations of IL-4 and IL-2 induce the production of large amounts of IgM and IgA.

Materials and Methods

Reagents. The anti-CD40 mAb 89 was produced in the laboratory (6). The CDw32/FcγRII-transfected Ltk⁻ cell line (CDw32 L cells) was kindly provided by Dr. K. Moore (DNAX, Palo Alto, CA) (10). Cell phenotype was determined using FITC-conjugated mAbs originating from Becton Dickinson & Co. (Mountain View, CA). The F(ab')₂ fragments of goat anti–human IgD and IgM were from Kallestad Laboratories, Inc. (Austin, TX). Cultures were carried out in modified Iscove's medium as detailed previously (11).

Cell Preparations and Cell Cultures. B cells were isolated from tonsils as described earlier (12). The isolated population expressed >98% CD19 or CD20 (B cells) and <1% CD2 (T cells) or CD14 (monocytes). Proliferation assays were performed in 96-well V-shaped microtiter plates (CEB, Nemours, France) in which 2.5 × 10³ irradiated (7,000 rad) CDw32 L cells were added. 2.5 × 10⁴ purified B cells were then added to cultures. Cells were pulsed with 1 μCi [³H]TdR usually at day 3 and 7. [³H]TdR uptake was measured by standard liquid scintillation counting techniques after harvesting. In most experiments, 5 × 10⁵ B cells in 1 ml were cultured on 2.5 × 10⁴ irradiated CDw32 L cells in 24-well plates, for Ig production. Supernatants were harvested after 10 d and Ig levels were determined by ELISA. Ig levels were also determined in the supernatants of cells that had been split at various time in-

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[1] *Abbreviations used in this paper:* Ag, antigen; CK, cytokine; GM, granulocyte/macrophage; rh, recombinant human.

Part of this work has been presented in Abstract forms at the 1990 UCLA Symposium on B lymphocyte development, March 31–April 6, Park City, UT (1, 2).

tervals. The production of IgG, IgM, IgA, and IgE was measured in standard ELISA as described elsewhere (11).

Cytokines. Each of the tested CKs was used over a wide range of concentrations. Purified recombinant human (rh) IL-1α (10^8 U/mg) was purchased from Genzyme (Boston, MA) and was usually used at 25 U/ml (2.5–150 U/ml). Purified rhIL-2 (3 × 10^6 U/ml) and rhIFN-γ (10^7 U/mg) were purchased from Amgen Biologicals (Thousands Oaks, CA) and were, respectively, used at 20 U/ml (1–50 U/ml) and 500 U/ml (25–1,250 U/ml). Purified rhIL-5 was provided by R. Coffman (DNAX). It was usually used at 15 ng/ml, which represents five times the concentration required for optimal growth and differentiation of human eosinophil precursors. Purified rhIL-3 (5 × 10^6 U/mg) and granulocyte/macrophage (GM)-CSF (2 × 10^6 U/mg) were provided by S. Tindall (Schering-Plough Research, Bloomfield, NJ) and were usually, respectively, used at 10 ng/ml (1–50 ng/ml) and 100 ng/ml (10–250 ng/ml), which were saturating for the growth of hematopoietic precursors in liquid cultures or in colony assays performed in semi-solid medium. Purified rhIL-4 (derived from *Escherichia coli*; 10^7 U/mg) was provided by P. Trotta (Schering-Plough Research). In most experiments, IL-4 was used at 100 U/ml (0.1–1,750 U/ml), which provides maximal stimulation of B cell growth in these culture conditions.

Purified *E. coli*–derived rhIFNα2b (2 × 10^9 U/ml; Schering-Plough Research) was usually used at 250 U/ml (50–1,000 U/ml). Purified rhTNF-α (2 × 10^7 U/mg; Genzyme) was usually used at 5 ng/ml (1–20 ng/ml). Purified rhIL-6 (10^7 U/mg; Genzyme) was usually used at 5 ng/ml (0.25–25 ng/ml). Human IL-7, purified from Cos 7 supernatant, was provided by F. Lee and J. Wideman (DNAX). It was usually used at 25 U/ml (1–100 U/ml), one unit being defined as the concentration inducing the half-maximal proliferation of the murine pre-B clone K.

Results

Proliferation of Anti-CD40-activated B Cells in Response to CKs. To study whether CKs would costimulate with cross-linked anti-CD40 to induce B cell proliferation, purified resting B cells were cultured on CDw32 L cells with 0.5 μg/ml mAb 89 with or without 11 different recombinant CKs, each of them used at various concentrations. [³H]TdR uptake was measured at day 3 and 7. Results illustrated in Fig. 1 were pooled from several independent experiments performed under exactly the same conditions (at day 7) and show the CK concentration that was found to be optimal in this assay. Results obtained with IL-4 alone represent the mean of 23 different experiments performed over an 18-mo period. The other CKs used alone or in combination with IL-4 have been tested in three to five independent experiments. As illustrated in Fig. 1 *A*, IL-4 strongly potentiated B cell proliferation. As shown in Fig. 2 *A*, the IL-4-induced [³H]TdR uptake was optimal at ~100 U/ml, but concentrations as low as 2.5 U/ml displayed significant activity. IL-1α, IL-6, and IFN-γ were also found to enhance [³H]TdR uptake, but it was always lower than that observed in IL-4. Their proliferative effects were dose dependent and were maximal at, respectively, 25 U/ml, 2 ng/ml, and 250 U/ml (data not shown). IL-2 and IL-3 also weakly enhanced [³H]TdR uptake, but IL-5, IL-7, TNF-α, GM-CSF, and IFN-α2b did not.

As IL-4 was reproducibly a strong enhancer of B cell growth, we tested whether the other CKs would modulate its effects.
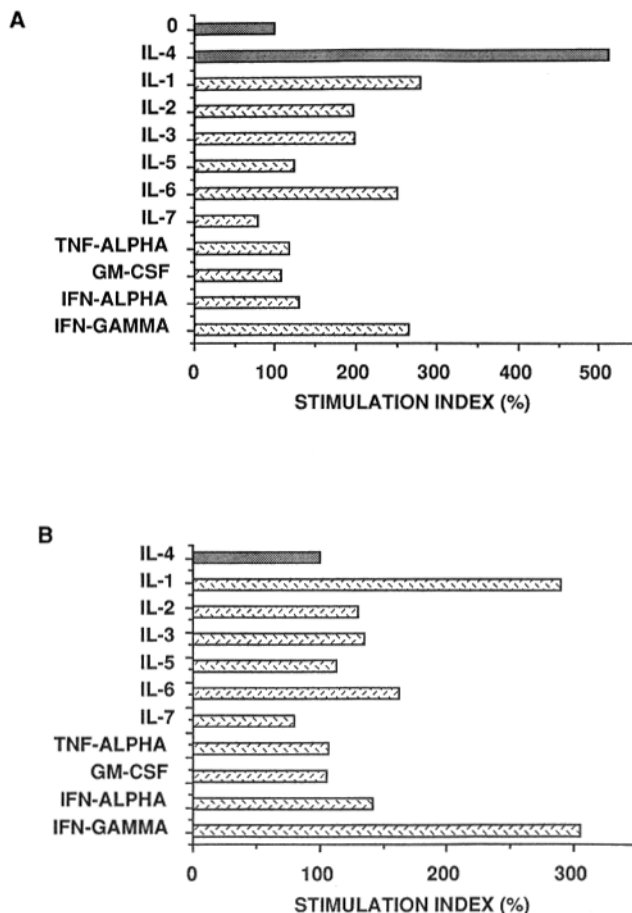
IL-1α and IFN-γ enhanced the [³H]TdR uptake induced by IL-4 (Fig. 1 *B*), and maximum stimulation was obtained with 25 and 250 U/ml, respectively. IL-2, IL-3, and IL-6 also slightly stimulated the [³H]TdR uptake induced by the combination of IL-4 and anti-CD40 but were less efficient than IL-1α or IFN-γ. All the other double CK combinations have been tested and the proliferation did not exceed those obtained with the most active CK of the pair (data not shown).

Combinations of IL-4 and anti-CD40 resulted in a 30–40-fold increase of the input B cells after 21 d. Whereas IL-1α only marginally enhanced the increase of B cells obtained with IL-4 and anti-CD40, IFN-γ further stimulated cell multiplication, and combinations of anti-CD40, IL-4, and IFN-γ
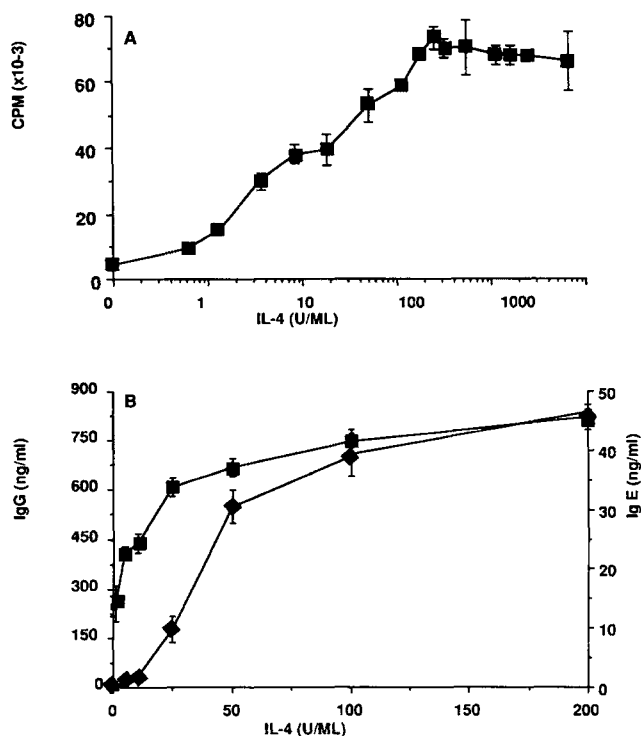
Figure 2. IL-4 induces anti-CD40-activated B cells to proliferate and produce Ig. (*A*) B cell proliferation: 2.5 × 10⁴ purified spleen B cells were cultured for 7 d on 2.5 × 10³ irradiated CDw32 L cells with 0.5 μg/ml mAb 89 and increasing concentrations of IL-4. Cells were pulsed with [³H]TdR for 16 h. Results are means ± SD of triplicates. (*B*) Ig production: 5 × 10⁵ B cells were seeded on 2.5 × 10⁴ irradiated CDw32 L cells without or with increasing concentrations of IL-4. Supernatants were harvested after 10 d and Ig levels were measured by ELISA. Representative of three experiments: (■) IgG (left ordonates): (◆) IgE (right ordonates) production. The IgM dose-response curve is similar to that of IgG (not shown).

Table 2. *IL-4 and Anti-CD40-dependent Normal Human B Cell Lines Produce Igs*

		Ig levels			
	IL-4	IgG	IgA	IgM	IgE
		ng/ml			
Day 7	–	10	<5	<10	<0.2
	+	630	8	40	18
Day 14	–	60	<5	<10	<0.2
	+	950	10	1,000	>30
Day 17	–	–	–	–	–
	+	450	30	40	>30
Day 21	–	–	–	–	–
	+	875	30	400	13

10⁵ purified B cells were cultured in 500 μl on 2.5 × 10⁴ irradiated CDw32 L cells in the presence of 0.5 μg/ml mAb 89 without or with 100 U/ml IL-4. Cell cultures have been split at the indicated times and 10⁵ cells were seeded on new CDw32 L cells and new medium. Ig levels in supernatants were determined by ELISA. Results are means of Ig levels in duplicate cultures. Cells cultured without IL-4 did not grow beyond 14 d and Ig levels were therefore not determined. Data are representative of five experiments.

resulted in a 80–90-fold expansion of input B cells (not shown). All the cells expressed, after 18 d, CD19, CD20, CD21, CD24, CD37, and HLA DR, but did not express CD3, CD4, or CD8 (data not shown).

Ig Production of B Cells Activated by Anti-CD40 and CKs. Purified B cells, seeded on CDw32 L cells, with or

Table 1. *Cytokine-induced Production of Igs by Anti-CD40-activated B Lymphocytes*

Ig	IL-4	Ig levels					
		0	IL-1	IL-2	IL-5	IL-6	IFN-γ
		ng/ml					
M	0	43	82	345	179	20	122
	+	1,194	698	11,856	420	520	964
G	0	433	330	637	402	602	578
	+	649	430	902	491	370	1,088
A	0	185	160	281	247	155	248
	+	276	520	2,500	863	118	1,115
E	0	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3
	+	58	63	59	47	84	49

10⁵ purified B cells were cultured in 500 μl on 2.5 × 10⁴ irradiated CDw32 L cells in the presence of 0.5 μg/ml mAb 89 without or with 100 U/ml IL-4 without or with the indicated CK. Supernatants were harvested after 10 d and Ig levels were determined by ELISA. Results are means of triplicate determinations. SD are not shown as they usually represent <10% of the mean value. Data are representative of three experiments.
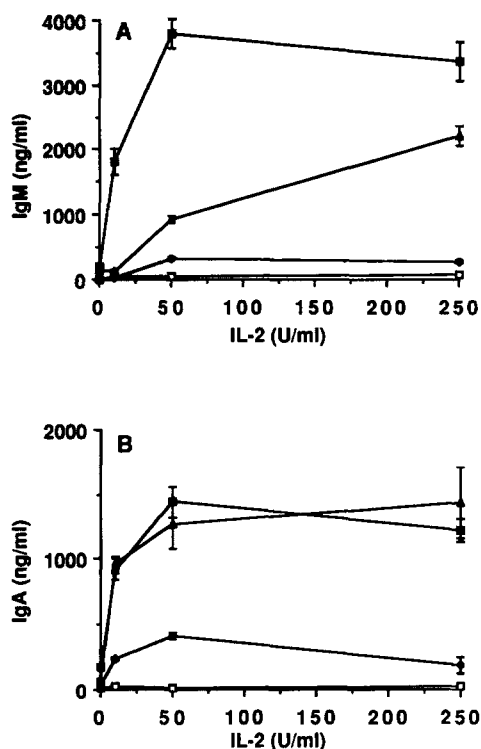
Figure 3. Combinations of IL-2 and IL-4 induce anti-CD40-activated B cells to produce IgM and IgA. 5 × 10^5 purified B cells were cultured on 2.5 × 10^4 irradiated CDw32 L cells in the presence of 0.5 μg/ml mAb 89 and increasing concentrations of IL-4 ([□] no IL-4; [◆] 10 U/ml; [▲] 50 U/ml; [■] 250 U/ml) and IL-2. Supernatants were harvested after 10 d and Ig levels were measured by ELISA. (*A*) IgM; (*B*) IgA. Data are representative of two experiments.

without mAb 89, and with or without recombinant CKs, were tested for their ability to produce Igs. As illustrated in Table 1, crosslinking of CD40 did not result in significant Ig production, although supernatants of cells cultured with mAb 89 contained a few-fold more IgM and IgG than those of cells cultured without mAb 89. IL-1α, IL-2, IL-3, IL-5, IL-6, and IFN-γ only marginally altered Ig production. However, IL-4 consistently stimulated IgM and IgG production. The most striking finding was the IL-4-induced production of IgE. As shown in Fig. 2 *B*, the IL-4-induced increase of Ig production was dose dependent, and the plateau was obtained for ~100 U/ml IL-4 for all three isotypes (data not shown, for IgM). The concentration of IL-4 inducing a half-maximal response was practically the same for the three isotypes (~10 U/ml for IgG and IgM; ~40 U/ml for IgE). B cells cultured for 21 d with anti-CD40 and IL-4 continuously produced the four isotypes (Table 2).

We also tested whether the IL-4-induced Ig production could be modulated by other CKs (Table 1). IL-1α, IL-3, IL-5, IL-6, and IFN-γ only weakly modulated Ig production induced by IL-4. Note in particular the lack of inhibitory effect of IFN-γ on IL-4-induced IgE production. Most interestingly, combinations of IL-2 and IL-4 resulted in production of IgM and IgA. As shown in Fig. 3, the induced IgM and IgA productions were a function of IL-2 concentration.

Discussion

Recently, we have reported that anti-CD40 antibodies presented on FcγRII/CDw32-transfected L cells can induce the proliferation of resting B lymphocytes (9). This system is unique in that it is the first one that allows an increase of the total number of seeded human B cells. The present study was aimed at determining how CKs affect the proliferation and differentiation of these anti-CD40-activated B cells. Among 11 tested recombinant CKs (IL-1α to 7, GM-CSF, TNF-α, IFN-α2b and IFN-γ), only IL-4 was found to strongly costimulate with anti-CD40 to induce B cell proliferation as measured both by [^3H]TdR uptake and cell counts. Unlike what was previously demonstrated with anti-IgM-activated B cells where IL-4 appears to act as a short-term growth factor (12), the activating property of IL-4 on anti-CD40-activated B cells was long lasting. IL-1α, IL-6, and IFN-γ were found to enhance [^3H]TdR uptake, but the effect was not sufficient to result in increased cell counts. IFN-γ, and to a lesser extent IL-1α, were found to enhance B cell proliferation induced by IL-4 and anti-CD40. The stimulatory effect of IFN-γ on IL-4/anti-CD40-induced B cell proliferation is in accordance with our earlier results showing that IFN-γ enhances the IL-4-induced proliferation of B cells costimulated with anti-IgM with or without IL-4 (12, 13). It is worth pointing out that IL-5 failed to enhance the proliferation of anti-CD40-activated B cells and, therefore, in contrast to murine IL-5 (14), human IL-5 has never scored positive in any of the assays measuring B cell proliferation (15). Likewise, TNF-α and IFN-α failed to induce the proliferation of anti-CD40-activated B cells, whereas they have been shown to induce the proliferation of B lymphocytes activated through their Ag receptors (16, 17). Also of interest is the virtual lack of growth-promoting activity of IL-2 on anti-CD40-activated B cells, whereas it is at least as potent and often more potent than IL-4 in inducing [^3H]TdR uptake in B cells stimulated with anti-IgM or *Staphylococcus aureus* Cowan (18). IL-3 was found repeatedly to provide a small proliferation signal to anti-CD40-activated B cells, which we also found with B cells costimulated with anti-IgM (T. Defrance and J. Banchereau, unpublished observations). Finally, as observed with B cells stimulated through their Ag receptor, IL-7 and GM-CSF were unable to induce the proliferation of anti-CD40-activated B cells.

B cells activated with anti-CD40 were found to produce relatively little IgM, IgG, and IgA. Whereas IL-1α, IL-2, IL-3, IL-5, IL-6, and IFN-γ did not enhance the Ig production of proliferating B cells, IL-4 was able to enhance IgM and IgG production and to induce the production of large amounts of IgE. This contrasts with previous studies showing the necessary role of T cells and monocytes in IL-4-induced differentiation of B cells to IgE production (19–21). However, a direct capacity of IL-4 to induce purified human B cells to produce IgE was recently described (22) where combined addition of EBV and IL-4 results in EBV-transformed B cell lines producing IgE. In our present system, we have been able to rule out a possible role for EBV by various means (9). The half-maximal concentrations of IL-4-inducing production of IgM, IgG, and IgE were similar. This contrasts with the con-

centrations of IL-4 required to induce LPS-activated murine B cells to produce IgE that are higher than those necessary to induce IgG (23). Interestingly, IFN-γ appears unable to inhibit the IL-4-induced IgE production by anti-CD40-activated human B cells, at variance with the EBV system (22), where IFN-γ can block the IL-4-independent generation of IgE-producing EBV cell lines. It also differs from the IL-4-induced IgE production by mouse LPS-activated B cells, which is also inhibited by IFN-γ (24). The lack of inhibitory activity of IFN-γ in IL-4-induced IgE production has, however, been observed with cord blood B cells (25). Taken together, these data suggest that IFN-γ may inhibit IL-4-induced IgE production by adult mononuclear cells in an indirect fashion. Our present observation suggests that triggering of CD40 may represent an important event in the IL-4-induced IgE production. It remains to be established whether the observed production of IgE results from the expansion of IgE-committed B cells or from an IL-4/anti-CD40-induced switch. We presently favor the latter hypothesis, as preliminary experiments have shown that naive IgD⁺ B cells do indeed produce IgE under these culture conditions. Our present finding raises the question as to whether the IgE production observed in an allogeneic T-B cell interaction (21, 26, 27) or in the IL-4 activation of mononuclear cells (19, 20, 27) involves triggering of the B cell CD40 Ag.

The combination of IL-4 and IL-2 resulted in a strong IgM

and IgA production by anti-CD40-activated B cells. It will be important to establish whether this results from the differentiation of IgA-committed B cells or from an induced isotype switch of naive B cells. Note that IL-5 does not induce anti-CD40-activated B cells to secrete IgA, although this CK has been shown to enhance IgA production of murine and human B lymphocytes in other culture systems (3). The presently tested combinations of CKs failed to result in a significant stimulation of IgG production, suggesting that other combinations of CKs will have to be tested or that other presently untested (and maybe not yet identified) CKs are involved in the production of IgG. This is particularly puzzling as IgG represents by far the major isotype in serum.

To conclude, this work shows that the combination of IFN-γ and IL-4 is, at the present time, the most potent one to induce the expansion of B cells activated through their CD40 Ag. Furthermore, it shows that anti-CD40-activated purified B cells produce IgE in response to IL-4 and that the further addition of IL-2 induces cells to secrete large amounts of IgA and IgM. Therefore, this new system represents a unique model to study isotype regulation in highly purified human B lymphocytes. Finally, the dramatic effects of CD40 ligation on B cell function make it important to determine the nature of the CD40 ligand, particularly since the CD40 Ag has been shown to display an important homology with nerve growth factor receptor (7) and TNF receptors (8).

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