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Activation of human B lymphocytes through CD40 and interleukin 4

We have produced and characterized a new CD40 monoclonal antibody, mAb 89, which in the presence of anti-IgM antibodies co-stimulates to induce B cell proliferation. mAb 89 activates resting B cells as shown by an increase in cell volume and an enhanced subsequent proliferation of B cells in response to anti-IgM antibody. However, mAb 89 does not prepare B cells to respond to the growth-promoting activity of interleukin (IL) 2 or IL 4. Unlike IL 2 and IL 4, mAb 89 only weakly stimulates the proliferation of anti-IgM pre-activated B cells. Thus, the activating properties of anti-CD40 are likely to explain its co-stimulatory effect on B cells.
Interestingly, the anti-CD40 mAb 89 was found to act in synergy with IL 4, but not with IL 2, in co-stimulation and restimulation assays. In this respect, anti-CD40 does not induce a significant increase of B cell surface IL 4 receptors while IL 4, but not IL 2, induces a twofold increase of the CD40 antigen expression. Thus the synergistic interaction between IL 4 and anti-CD40 may be related to the IL 4-dependent increase of CD40 antigen expression.

1 Introduction

Schematically, maturation of resting B cells into plasma cells occurs via three successive steps: activation, proliferation and differentiation (reviewed in [1, 2]). Along with lymphokines, mAb with agonistic properties represent powerful tools to study the different steps of B cell maturation (reviewed in [3, 4]). A number of agents were recently identified which allow progression of the B cells through these different steps. B cell activation can be induced by antigen, by suboptimal concentrations of surrogate antigens (anti-IgM and anti-IgD antibodies), by the mAb 1F5 against the CD20 antigen [5] and mAb G28-8-specific for the B cell surface glycoprotein Bgp95 [6], or by certain lymphokines like IL 4 [7] or by B cell activating factor (BCAF; [8, 9]). Proliferation of activated B cells can be induced by several lymphokines like IL 2 [10], IL 4 [11], IFN [12–14], low molecular weight B cell growth factor (BCGF; [15], high molecular weight BCGF [16–18], TNF-α [19] and -β [20], as well as certain mAb directed against CD21 [21, 22], CD22 [23], CD23 [24, 25] and CD40 [26–28]. Finally, B cell differentiation can be induced by several lymphokines like IL 2 [29], IL 4 [30, 31], IL 6 [32], as well as certain mAb against the C3 receptors like CR1 [33] or CR2 [22].

With the final goal of establishing mAb specific for the receptors of B cell growth and activation factors, we undertook a study aimed at producing mAb antibodies mimicking the effects of B cell growth and activation factors. During the course of this study, we produced a new anti-CD40 monoclonal antibody, mAb 89, which activates B cells and which in the presence of anti-IgM antibodies co-stimulates to induce B cell proliferation. IL 4 and mAb 89 were found to act in synergy to induce B cell activation and proliferation. Whereas anti-CD40 did not affect significantly IL 4R expression on B cells, IL 4 was found to enhance the expression of the CD40 antigen.

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2 Materials and methods

2.1 Reagents, antibodies and lymphokines

mAb 89 was produced, according to standard protocols [34], by immunizing mice with anti-IgM antibody-activated tonsillar B cells. mAb 89 was isolated from ascites fluid by HPLC with an anion-exchange column (DEAE 5PW; Waters Associates, Millipore Corp., Milford, MA). *Staphylococcus aureus* Cowan strain I (SAC) and anti-IgM antibody coupled to beads (Immunobeads) were purchased from Hoechst-Calbiochem (La Jolla, CA) and Bio-Rad (Richmond, CA), respectively. FITC-conjugated $F(ab')_2$ fragments of goat anti-mouse Ig were from Grub (Vienna, Austria). The anti-CD40 mAb G28-5 (kindly provided by Dr. E. A. Clark, University of Washington, Seattle, WA) was described earlier [26, 27]. Purified rIL 2 (3×10^6 U/mg) was from Amgen (Thousand Oaks, CA). Purified rIL 4 (10^7 U/mg) was from Schering Plough Research (Bloomfield, NJ).

2.2 Cell sources, culture conditions and flow cytometry

Tonsil B cells were isolated from tonsils as previously described [30]. The tonsillar B cell preparation contained >95% B cells, <1% T cells and <1% monocytes as determined by specific mAb (anti-CD20, anti-CD2, anti-CD15). Purified B cells were separated into *in vivo* pre-activated B cells (low density) and resting B cells (density >1.075) using a discontinuous gradient of Percoll (Pharmacia, Uppsala, Sweden; [30]). 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, St. Louis, MO) as described by Yssel et al. [35]. Furthermore, 2% heat-inactivated FCS (Industrie Biologique Française, Genevilliers, France) was added to the medium. For co-stimulation assay with insolubilized anti-IgM antibody, 5×10^4 purified B cells were cultured in 96-well microtrays (Falcon, Oxnard, CA) in the presence of insolubilized anti-IgM antibody (5 μg/ml) in a final volume of 100 μl. Reagents to be tested for co-stimulatory activity were added at the initiation of the culture. Cells were pulsed with [^{3}H]dThd (1 μCi = 37 kBq/well, specific activity: 25 Ci/mmol, CEA, Saclay, France) during the last 16 h of a 3-day culture period.

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[^{3}H]dThd uptake was measured by standard liquid scintillation counting techniques after harvesting. For restimulation assay with pre-activated B cells, high-density purified B lymphocytes, adjusted at 2×10^6 cells/ml in complete medium (described above), were stimulated by insolubilized anti-IgM antibody (5 μg/ml) for 24 h. After activation, B cell blasts were harvested, and centrifuged over Percoll gradient to remove nonviable cells as well as anti-IgM beads. For detection of BCGF activities, fresh B cell blasts, resuspended at 1×10^6 cells/ml in complete medium, were dispensed under a volume of 50 μl into wells of microtiter trays. Reagents and supernatants to be tested were added to reach a final volume of 100 μl. B cell proliferation was assessed by measurement of [^{3}H]dThd uptake during the last 16 h of culture. The proliferative response of B cell blasts to BCGF was routinely measured on day 3. Fluorescence staining was performed in microtrays as described earlier [34] and staining was analyzed with a FACScan (Becton Dickinson, Sunnyvale, CA).

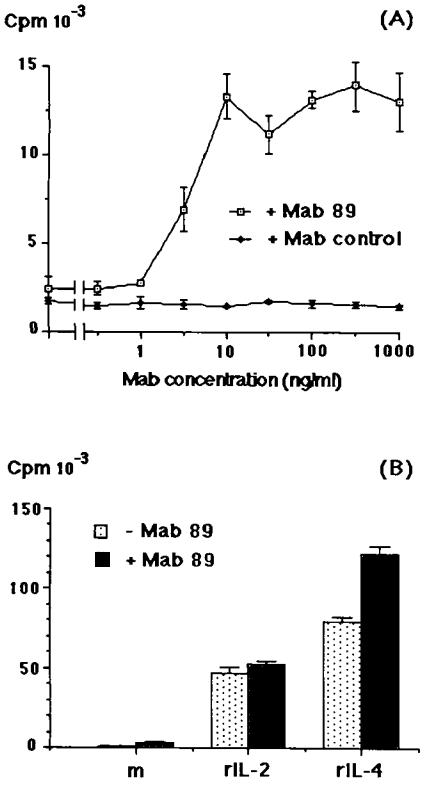

Figure 1. mAb 89 enhances B cell proliferation. (A) Co-stimulatory effects of mAb 89 and anti-IgM antibody on B lymphocytes. Tonsillar B lymphocytes (5×10^4) were cultured for 72 h with 5 μg/ml insolubilized anti-IgM antibody and increasing concentrations of purified mAb 89. Cells were labeled with [^{3}H]dThd for the last 16 h of the culture period. The IgG_1 mAb used as a control is specific for denatured human IFN-γ. Results are mean ± SD of triplicate determinations. The experiment is representative of three. (B) mAb 89 does not significantly induce proliferation of pre-activated B cells but strongly enhances their IL4-induced proliferation. High density tonsillar B cells (2×10^6/ml) were cultured with insolubilized anti-IgM antibody (5 μg/ml) for 24 h. Viable cells recovered on a Percoll gradient were cultured (5×10^4 in 100 μl) for 3 days with the appropriate stimulus. [^{3}H]dThd incorporation was measured during the last 16 h of the culture period. Data are expressed as the mean ± SD of triplicate determination. This experiment is representative of four.

2.3 Equilibrium binding experiments

Experiments were carried out as described elsewhere [36]. Purified IL4 was iodinated according to the Tejedor-Ballesta method without significant loss of biological activity. B cells were washed twice and resuspended in cold RPMI 1640, 2% BSA, 20 mM Hepes, pH 7.2 (binding medium). Cell aliquots (3×10^6 cells/0.5 ml) were incubated for 4 h at 4 °C in the presence of various iodinated IL4 concentrations. Surface bound iodinated IL4 was then separated from unbound iodinated IL4 by centrifugation (250 × g, 10 min at 4 °C). Supernatants were recovered for measurement of unbound (free) radioactivity whereas cell pellets were washed twice before measurement of surface-bound radioactivity. Levels of radioactivity in both samples were measured in a gamma counter. Nonspecific binding was determined in the presence of 10 nM unlabeled IL4.

3 Results

3.1 mAb 89, a new agonistic CD40 mAb

Hybridoma clone 89, which produces the mAb 89 (IgG_1), was selected because its supernatant specifically stained purified B cell populations (data not shown) and because its supernatant strongly enhanced the proliferation of B cells co-stimulated with anti-IgM antibody (Fig. 1A). The co-stimulatory effect was observed at very low concentrations of the antibody, since 10 ng/ml (50 pM) produced maximal activation. The stimulation index (ratio of cpm in the presence of anti-IgM antibody and mAb 89 : cpm in the presence of anti-IgM antibody) was consistently between 3 and 6. Immunoblotting experiments showed that mAb 89 binds to a 45–50-kDa antigen expressed on B cells (not shown). The co-stimulatory effect of mAb 89, the pattern of binding to various lymphoid cells, and the molecular weight of antigen 89 led us to hypothesize that mAb 89 was recognizing the CD40 antigen. Competition studies showed that the G28-5 mAb [27], which allowed definition of the CD40 cluster together with the mAb S2C6 [28], was able to totally inhibit the binding of biotinylated mAb 89 to B cells (data not shown). Thus, mAb 89 is specific for the CD40 antigen.

Table 1. Proliferation of anti-CD40 pre-activated B cells in response to anti-IgM antibody, rIL4 or rIL2[a)]

Stimulus	[^{3}H]dThd incorporation (cpm × 10^{-3}) Control mAb	mAb 89
0	0.8 ± 0.1	2.5 ± 0.3
Anti-IgM (5 μg/ml)	5.3 ± 0.3	24.0 ± 1.7
rIL4 (250 U/ml)	2.5 ± 0.4	7.4 ± 0.3
rIL4 + anti-IgM	12.4 ± 0.4	36.9 ± 2.0
rIL2 (10 U/ml)	4.1 ± 0.3	8.8 ± 0.6
rIL2 + anti-IgM	17.4 ± 3.1	45.0 ± 1.4

a) High density purified tonsillar B cells (2×10^6/ml) were stimulated or not with 1 μg/ml mAb 89 or an IgG_1 mAb specific for denatured human IFN-γ used as a control for 48 h. Viable cells recovered on Percoll gradient were cultured (5×10^4 in 100 μl) for 3 days. [^{3}H]dThd incorporation was measured during the last 16 h. Data are expressed as the mean ± SD of triplicate determinations. This experiment is representative of two.

3.2 Kinetic study of the anti-CD40 co-stimulatory effects

Kinetic experiments were designed to elucidate whether the co-stimulatory effect of mAb 89 was due to an effect of anti-CD40 on the earlier stages of B cell activation and/or on the later stages of B cell activation, once B cells have received activation signals from the anti-IgM antibody. Thus, resting B cells were preactivated for 24 h with insolubilized anti-IgM antibody and viable B cell blasts were recultured in the presence of mAb 89 with or without IL2 or IL4. Data illustrated in Fig. 1B demonstrate that mAb 89 only induced a weak proliferation of anti-IgM pre-activated B cell blasts, whereas IL2 and IL4 were very efficient. This indicates that anti-CD40 does not act as a progression factor. Interestingly, mAb 89 consistently enhanced the IL4-dependent B cell proliferation, but did not significantly alter the IL2-dependent proliferative response of these B cell blasts. Likewise, mAb 89 synergized with IL4 but not IL2 in a co-stimulation assay with anti-IgM antibody (data not shown). Preculturing B cells for 48 h with mAb 89 was insufficient to render them susceptible to the growth-promoting effect of IL2 or IL4 (Table 1). However, these mAb 89-pre-activated B cells displayed an enhanced proliferation in response to anti-IgM antibody used alone or in combination with IL4 or IL2. This enhancing effect was not due to an increase of surface IgM expression on B cell (data not shown).

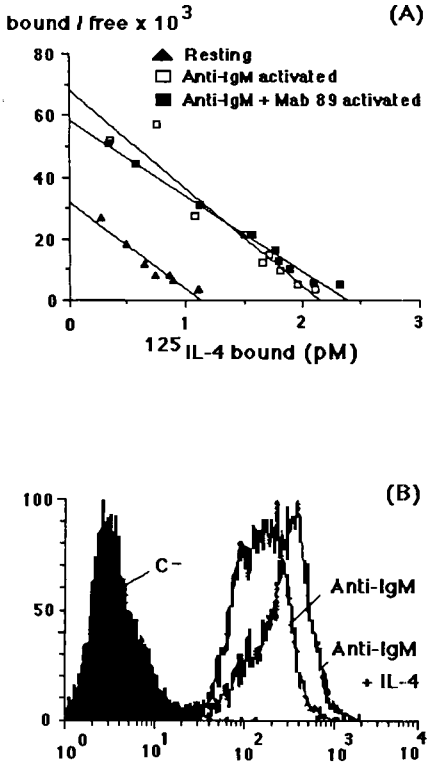

Figure 2. mAb 89 does not affect IL4R expression on B cells but IL4 up-regulates CD40 expression. (A) mAb 89 does not significantly affect the binding of iodinated IL4 to B cells. Binding of iodinated IL4 was determined on freshly isolated tonsil B cells (▲—▲) or purified tonsil B lymphocytes (2×10^6/ml) cultured for 40 h with anti-IgM antibody (10 μg/ml), with (■—■) or without (□—□) mAb 89 (5 μg/ml). Viable cells (3×10^6) were incubated for 4 h at 4 °C with various concentrations of iodinated IL4 as described in Sect. 2.3. Scatchard analysis of these binding data are plotted. This experiment is representative of three. (B) IL4 up-regulates the expression of CD40 antigen. Tonsillar B cells were cultured for 48 h with anti-IgM antibody, with or without IL4 (250 U/ml). Viable cells were stained with mAb 89, then with FITC-conjugated anti-mouse Ig. Dark histograms (C) represent background staining where an anti-denatured IFN-γ mAb was used. Abscissa: log fluorescence intensity. Ordinate: relative cell number. This experiment is representative of three.

3.3 Anti-CD40 induces an increase of B cell volume

The priming effect of anti-CD40 described in the previous paragraph led us to investigate whether mAb 89 would activate B cells. Thus, high density B cells were cultured for 48–72 h with mAb 89 or IL4, and variations of cell volume as well as induction of CD23 expression were monitored as markers of activation. mAb 89 was found to induce a slight but reproducible increase of B cell size which was comparable to that obtained with IL4 (Table 2). Increase of cell volume was homogeneous with both mAb 89 and IL4, and affected the majority of B cells (data not shown). Interestingly, combinations of IL4 and mAb 89 resulted in an increase of B cell volume which was more important than that observed with each factor alone. However, in contrast to IL4 [37], mAb 89 did not induce CD23 expression on B cells nor enhanced the CD23 expression induced by IL4, alone or in co-stimulation with anti-IgM antibody (data not shown).

3.4 Anti-CD40 does not significantly alter IL4R expression but IL4 enhances CD40 expression on B cells

We wondered whether the synergistic effects of anti-CD40 and IL4 on B cell proliferation were due to either an anti-CD40-mediated increase of IL4R and/or to an IL4-mediated increase of the CD40 antigen expression. Thus, resting B cells were cultured for 40 h with or without anti-IgM antibody, with or without anti-CD40, and iodinated IL4 binding was determined. Scatchard analysis of equilibrium binding data (Fig. 2A) demonstrated that anti-IgM-stimulated B cells reproducibly express 1.5- to 3.0-fold more IL4-binding sites than freshly isolated B cells or B cells cultured without any stimulant (Zuber, C. E. and Banchereau, J., manuscript in preparation). However, the anti-CD40 mAb 89 was unable to significantly increase the number and affinity of IL4R on anti-

Table 2. Anti-CD40 increases the size of resting B cells[a]

Stimulus	Volume in μm³
Control mAb[b]	170 ± 2
mAb 89 (300 ng/ml)	201 ± 2
rIL4 (250 U/ml)	197 ± 7
mAb 89 + rIL4	260 ± 4
SAC (0.05%)	326 ± 4
Isolated B cells[c]	167

a) High density purified tonsillar B cells (2×10^6) were cultured for 72 h in 2 ml complete medium with or without the appropriate stimulus. The mean volume of the cells was determined using a Coulter Counter coupled to a Channelyser. Each determination was performed in triplicate and results are expressed as mean ± SD. This experiment is representative of four.
b) The control mAb (IgG_1) is specific for human denatured IFN-γ.
c) Volume of the freshly isolated B cells.

IgM-costimulated B cells. The equilibrium dissociation constants for non-activated B cells, anti-IgM antibody-activated B cells and anti-IgM plus mAb 89-activated B cells were, respectively: 31 pM, 30 pM and 34 pM. The number of IL4-binding sites per cell for non-activated B cells, anti-IgM antibody-activated B cells and anti-IgM plus mAb 89-activated B cells were, respectively: 283, 530 and 591. mAb 89 also failed to modulate IL4R expression on resting B cells (data not shown).

Then, the effect of IL4 on CD40 antigen expression was studied. Tonsillar B lymphocytes were cultured for 48 h with insolubilized anti-IgM antibody, with or without IL4 or IL2, then stained with mAb 89 and finally analyzed by flow cytometry. As illustrated in Fig. 2B, IL4 enhanced the expression of CD40 antigen, by approximatively twofold, on anti-IgM antibody-co-stimulated B cells as well as resting B cells (not shown). IL2 did not increase the density of CD40 antigen expression on B cells (data not shown). Taken together, these data suggest that the synergistic effects of IL4 and anti-CD40 on B cell proliferation are likely to be related to the IL4-induced increase of CD40 antigen expression.

4 Discussion

In this report, we describe the activating effects of a novel CD40 mAb, mAb 89, on human B cells. mAb 89 recognizes an epitope on the CD40 antigen, which is identical or close to the epitope recognized by the mAb G28-5 [26, 27]. mAb 89, like mAb G28-5 [26], co-stimulates with anti-IgM antibody to induce B cell proliferation. On a molar basis, mAb 89 is as efficient as IL2 or IL4 since maximal effects were obtained in the 10^{-10} M concentration range. Resting B cells pre-incubated with anti-CD40, when compared to B cells pre-incubated with a control mAb, displayed an enhanced proliferation in response to anti-IgM antibody used alone or in combination with IL2 or IL4. In contrast, anti-CD40 displayed only minimal growth-promoting effects on anti-IgM antibody-pre-activated B cells. These data indicate that anti-CD40 activates resting B cells and this is likely to explain the costimulatory effect of mAb 89 on anti-IgM antibody-co-stimulated B cells. The activating capacity of anti-CD40 is further confirmed by its ability to increase the volume of resting B cells. Thus, anti-CD40 shares activating and co-stimulatory properties with IL4. However, anti-CD40 and IL4 differ in their mode of action, since (a) anti-CD40 and IL4 act in concert to induce the increase of B cell volume and the proliferation of anti-IgM antibody-co-stimulated or pre-activated B cells; (b) anti-CD40, unlike IL4 [37], is unable to induce FcεR2/CD23 expression on resting or activated B cells; (c) anti-CD40, unlike IL4 ([38], our unpublished observations), is unable to increase surface IgM expression on B cells; (d) unlike IL4, anti-CD40 only poorly induces the proliferation of anti-IgM antibody-pre-activated B cells and (e) anti-CD40 does not affect binding of iodinated IL4 to its receptor.

As described with phorbol ester and ionomycin pre-activated B cells [39], anti-CD40 and IL4 were found to synergize in a variety of B cell assays. In contrast, anti-CD40 was not found to significantly enhance the co-stimulatory effect of IL2 nor the growth-promoting effect of IL2. The current data indicates that the synergy between anti-CD40 and IL4 on B cells does not occur at the level of IL4R since anti-CD40 neither up-regulated the number nor the affinity of IL4R expressed on B cells. In contrast, IL4 was found to enhance the expression of CD40 antigen both on resting ([40], our unpublished observation) and anti-IgM-co-stimulated B cells, whereas IL2 was inefficient. Thus, the data suggest that the co-stimulatory effects of anti-CD40 and IL4 are likely to be related to the IL4-induced up-regulation of CD40. However, we cannot exclude that these co-stimulatory effects of anti-CD40 and IL4 may also occur within the cell through activation of complementary biochemical pathways. At the present time, the biochemical pathways involved in the activation of B lymphocytes by anti-CD40, as well as IL4, remain to be elucidated.

It will be of major interest to elucidate the biological function of the CD40 antigen. If it is a cytokine receptor, as proposed earlier [27, 28], then combinations of IL4 and the putative CD40 ligand are likely to play an important role in B cell activation and proliferation.

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