

## **B Cell Activation via CD40 Is Required for Specific Antibody Production by Antigen-stimulated Human B Cells**

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### **Summary**

Costimulatory signals provided by T cells are required for B cells to produce specific antibody (Ab) to T-dependent antigen (Ag) bacteriophage  $\phi$ x 174. In this study, we demonstrate that if cultured in the presence of anti-CD40, interleukin 10 (IL-10), and Ag, purified B cells can produce antiphage Ab in quantities comparable to those synthesized by B cells cocultured with Ag and T cells. Isotypes produced by B cells in this culture system correspond to those observed in sera of B cell donors. Culture of immunoglobulin (Ig)D<sup>-</sup> and IgD<sup>+</sup> B cells reveals that Ag-induced production of antiphage Ab is restricted to IgD<sup>-</sup> subset of B cells. In the absence of Ag, anti-CD40/IL-10-stimulated B cells produce only minute amounts of antiphage Ab, indicating that Ag stimulation is indispensable and provides a signal that is synergistic with anti-CD40 and IL-10. Addition of a soluble form of the CD40 ligand (sgp39) to the culture system has a similar effect on specific Ab synthesis as anti-CD40; addition of the soluble construct, CD40 Ig, known to inhibit gp39/CD40 interaction, suppresses *in vitro* antiphage Ab production by Ag exposed peripheral blood mononuclear cells. Finally, *in vivo* requirement of gp39/CD40 interaction for specific Ab production was demonstrated by the finding that activated T cells from patients with x-linked hyper IgM syndrome express functionally defective gp39 and respond with depressed Ab titers and fail to switch from IgM to IgG after multiple phage immunizations. These observations illustrate that *in vitro* and possibly *in vivo* Ag-specific Ab synthesis requires the presence of Ag and IL-10, and activation signals via CD40.

Helper T cells provide stimulatory signals that induce B cell proliferation and differentiation into Ig-producing cells. These stimulatory signals are transmitted through either cognate or noncognate mechanisms (1). Cognate interaction, which requires direct contact between B and T cells, provides the initial signals for B cell activation. Cytokines produced by helper T cells are known to transmit a second signal by noncognate mechanisms. Both cognate and noncognate stimuli are necessary to activate and differentiate resting B cells into terminal Ig-secreting cells (1).

Recently, two molecules, CD40 and CD40 ligand (gp39), involved in a cognate help were identified on B cells (CD40) and on activated T helper cells (CD40L, gp39) (2-4). The importance of CD40 for B cell activation was underscored by demonstrating that anti-CD40, as well as a soluble recombinant form of gp39, initiates B cell proliferation and differentiation into Ig-producing cells in the presence of cytokines (5-7). If cultured with IL-4, anti-CD40-activated B cells produce IgE and IgG4, while in the presence of IL-10 they syn-

thesize IgG, IgA, and IgM (5, 6). Thus, the combination of anti-CD40 and cytokines provide both cognate and noncognate stimulatory signals, which can substitute for T cell help to B cells. However, the role of CD40 and these cytokines in the Ag-specific Ab production is not well understood. To investigate this, we used the T cell-dependent Ag, bacteriophage  $\phi$ x 174 (phage) (8-10), which allows us to study the induction and regulation of phage-specific Ab synthesis.

### **Materials and Methods**

**Reagents.** Purified anti-CD40 mAb (G28-5 [IgG1]) was kindly provided by Dr. Edward A. Clark (Seattle, WA) (2). Purified human rIL-4 and rIL-10 were gifts from Dr. K. W. Moore, Dr. H. Ishida, and Dr. M. Howard (DNAX, Palo Alto, CA). The soluble fusion protein of the extracellular domain of human CD40 coupled with the Fc domain of human IgG1 (CD40 Ig), the soluble form of Leu-8 coupled with the same Ig fragment (Leu-8 Ig), (11) and supernatants of COS cells expressing soluble gp39 (sgp39) or soluble CD72 (sCD72) were generated as previously described (7).

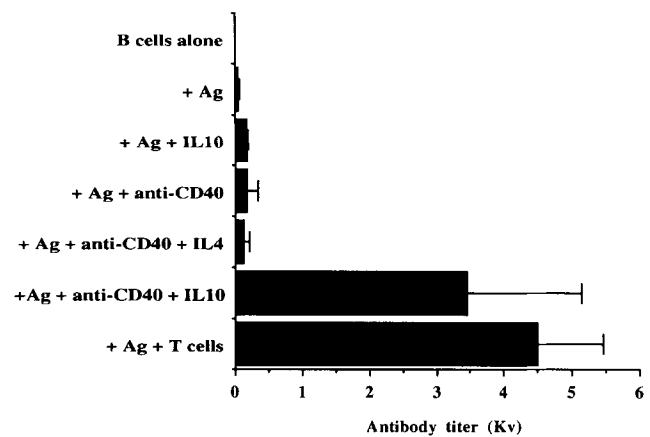
**Cell Preparations and Cell Cultures.** Highly purified human peripheral blood B cells and T cells were isolated as previously described (12). To obtain IgD<sup>-</sup> and IgD<sup>+</sup> B cells, purified B cells stained with FITC-conjugated anti-human IgD (Tago, Inc., Burlingame, CA) and PE-conjugated anti-CD19 (Leu-12; Becton Dickinson & Co., Mountain View, CA) were sorted into IgD<sup>-</sup> Leu-12<sup>+</sup> and IgD<sup>+</sup> Leu-12<sup>+</sup> B cells by FACStar<sup>®</sup> (Becton Dickinson & Co.). The final purity of both subsets was >99.7%.

B cells were cultured in 96-well plates at a final volume of 200  $\mu$ l in RPMI 1640 supplemented with 10% FCS (HyClone Laboratories, Logan, UT), 2 mM glutamine, 50 U/ml penicillin, and 50  $\mu$ g/ml of streptomycin. The cell concentration of B cells was  $2.5 \times 10^5$ /ml; in some experiments B cells were cultured in the presence of  $10^6$ /ml of autologous T cells. mAb G28-5 (final concentration, 1  $\mu$ g/ml), soluble gp39, soluble CD72 (diluted 1:4), IL-10 (10 ng/ml), and phage ( $10^6$  PFU/ml) were added on day 0. In the blocking experiments, CD40-Ig or Leu-8 Ig was added on day 0 at various concentrations. After 12 d, the supernatants were collected and tested for Ig concentrations by ELISA (13) and phage-neutralizing Ab activity (8).

**Bacteriophage  $\phi$ x174, Immunization Protocol, and Ab Determination.** A single lot of phage prepared as previously described (8) was used for in vivo immunizations and for the in vitro experiments. Human volunteers and four patients from three families with x-linked hyper IgM syndrome were injected intravenously twice, 6 wk apart, with phage at the standard dose of  $2 \times 10^9$  PFU/kg body weight. Serum was collected before and at weekly intervals after immunizations and antiphage Ab activity determined by a sensitive phage neutralization assay, expressed as the rate of phage inactivation (K value,  $Kv$ ) (8). Using this method, the lower limit of sensitivity in detecting phage-neutralizing Ab is a K value of 0.01. Susceptibility of phage-neutralizing Ab to 2-ME was determined by the method of Grubb and Swahn (14); neutralizing Ab resistant to 2-ME was considered to be IgG.

## Results

**Production of Antiphage Ab by Human B Cells.** Purified B cells obtained from human subjects 6 wk or later after the last immunization with phage failed to produce spontaneous antiphage Ab in the absence of Ag, indicating that they were in a resting stage (Fig. 1). Addition of Ag (phage) to cul-



**Figure 1.** Antiphage Ab production by purified human B cells. B cells ( $0.5 \times 10^5$ /well) were cultured alone or with various combinations of Ag (phage), IL-10, anti-CD40, or autologous T cells. Mean and 1 SD of triplicate cultures are shown.

tured B cells failed to induce phage-specific Ab production. Autologous T cells efficiently initiated the production of antiphage Ab by Ag-stimulated B cells, demonstrating that T cell help is necessary for the induction of phage-specific Ab production. Ag-stimulated B cells, in the absence of T cells and cocultured with a combination of anti-CD40 and IL-10, produced amounts of antiphage Ab similar to cultures to which autologous T cells were added. Neither anti-CD40 nor IL-10 alone induced specific Ab production by Ag-stimulated B cells, indicating that combination of anti-CD40 and IL-10 is required for specific Ab production. IL-4, known to induce anti-CD40-stimulated B cells to proliferate and to polyclonally produced Ig (5), had no effect on phage-specific Ab production.

The requirement of Ag for specific Ab production is shown in Table 1. In the absence of Ag, B cells cultured with anti-CD40 and IL-10 produced only minute amounts of antiphage

**Table 1.** Effect of Ag Stimulation on the Production of Antiphage Ab

Cells	Stimulation		Antiphage Ab titer		
	Ag (phage)	anti-CD40 + IL-10	Exp.1	Exp.2	Exp.3
B cells	None	None	<0.01	<0.01	<0.01
	None	+	$0.17 \pm 0.004$	$0.02 \pm 0.005$	$0.02 \pm 0.002$
	+	None	$0.03 \pm 0.031$	$0.03 \pm 0.019$	$0.17 \pm 0.004$
	+	+	$4.24 \pm 1.311$	$2.70 \pm 0.676$	$2.20 \pm 0.865$
B + T cells	None	None	<0.01	<0.01	<0.01
	+	None	$4.50 \pm 0.670$	$3.26 \pm 0.544$	$8.38 \pm 2.531$

Purified human B cells, or B and autologous T cells, were cultured for 12 d with various combinations of Ag (phage) or anti-CD40 and IL-10. Data are shown as mean  $\pm$  SD of the results obtained from triplicate cultures.

**Table 2.** Antiphage Ab Titers and Isotypes in Sera and Culture Supernatants

	Primary immunization		Secondary immunization	
	Donor 1	Donor 2	Donor 1	Donor 2
	<i>K<sub>v</sub></i>			
Sera	12.02 (0.8)*	7.61 (0.3)	158.24 (86.1)	118.65 (81.8)
Culture supernatants†	0.64 (0.7)	0.50 (0)	2.85 (65.0)	1.59 (87.4)

Peripheral blood was collected from two human volunteers 6 wk after primary or secondary immunization.

\* Total neutralizing Ab titer (percent IgG).

† B cells were cultured for 12 d with anti-CD40 and IL-10 in the presence of phage Ag. Means of triplicate cultures are shown. SD <15% of the mean for each point.

**Table 3.** Antiphage Ab Synthesized by IgD<sup>+</sup> and IgD<sup>-</sup> B cells

Cells	Stimulation	Total IgM	Total IgG	Antiphage Ab
		<i>ng/ml</i>	<i>ng/ml</i>	<i>K<sub>v</sub></i>
IgD <sup>+</sup> B cells	None	<30	<30	<0.01
	Anti-CD40 + IL-10	372 ± 48	<30	<0.01
IgD <sup>-</sup> B cells	None	40 ± 1	314 ± 44	<0.01
	Anti-CD40 + IL-10	188 ± 12	1,790 ± 647	2.59 ± 0.99*

Human peripheral blood B cells obtained from a fully immunized volunteer (serum *K<sub>v</sub>* = 98.0 [52% IgG and 48% IgM]) were sorted into Leu-12<sup>+</sup> IgD<sup>+</sup> cells and Leu-12<sup>+</sup> IgD<sup>-</sup> cells by FACStar<sup>®</sup>. The cells were cultured with or without anti-CD40 and IL-10 for 12 d in the presence of Ag (phage). Data are shown as mean ± SD obtained from triplicate cultures. Similar results were obtained by three independent experiments.

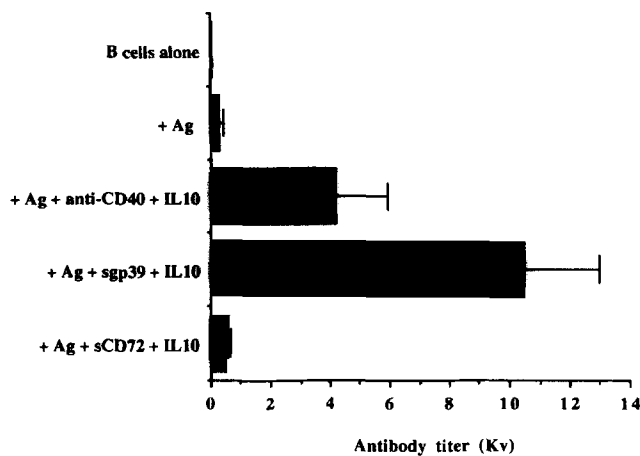
\* The phage-specific Ab consisted of IgG (56%) and IgM (44%).

Ab. Similarly, B cells cultured with autologous T cells in the absence of Ag failed to produce detectable antiphage Ab. However, addition of Ag to B cells cultured in the presence of anti-CD40 and IL-10 resulted in the production of high titers of phage-neutralizing Ab, reaching *K* values (*K<sub>v</sub>*) that were comparable (26–94%) to those of B cells cultured in the presence of Ag and autologous T cells.

**Isotypes of Antiphage Ab Produced by Human B Cells.** Serum antiphage Ab produced in response to a primary immunization with phage consists mainly of the IgM isotype and in response to a secondary immunization, when class switch has occurred, mainly of the IgG isotype (8, 9) (Table 2). We measured the IgG proportion of in vitro antiphage Ab produced by B cells after primary and secondary immunization. Phage-specific Ab produced in vitro by anti-CD40/IL-10-stimulated B cells obtained from volunteers after primary immunization was predominantly of the IgM isotype, and after secondary immunization, predominantly of the IgG isotype, reflecting the isotype of phage-specific Ab observed in the B cell donor's serum. These results indicate that IL-10 has very little effect, if any, on heavy chain switching, but has a profound effect on the amount of Ab produced by Ag-specific B cells.

**Production of Antiphage Ab by IgD<sup>-</sup> B Cells.** B cells obtained from a fully immunized volunteer were further separated into Leu-12<sup>+</sup> IgD<sup>+</sup> cells and Leu-12<sup>+</sup> IgD<sup>-</sup> cells using flow cytometry. Serum antiphage Ab of the twice-immunized human donor used in this experiment consisted of 52% IgG and 48% IgM. As shown in Table 3, IgD<sup>-</sup> B cells synthesized antiphage Ab if cultured in the presence of Ag, anti-CD40, and IL-10, indicating that highly purified B cells can synthesize Ag-specific Ab in this culture system. The antiphage Ab produced in vitro was 56% IgG and 44% IgM, demonstrating that both IgG- and IgM-committed cells in IgD<sup>-</sup> B cells were activated under these conditions. In contrast, IgD<sup>+</sup> B cells failed to produce antiphage Ab although they responded to stimulation with anti-CD40 and IL-10 by increased production of total IgM. These results confirm the observation by Jelinek et al. (15) that Ag-induced memory B cells are contained in the IgD<sup>-</sup> subset and that naive B cells are part of the IgD<sup>+</sup> subset.

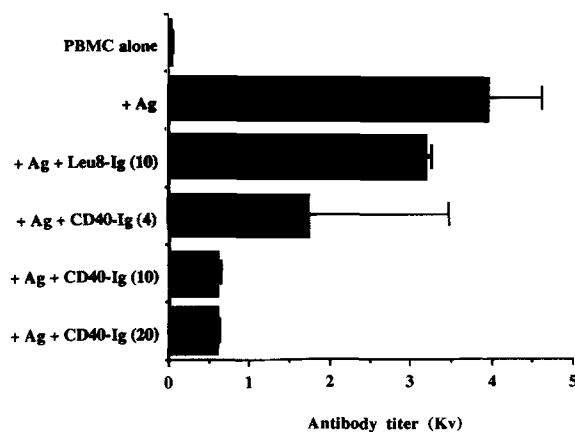
**Effect of Soluble gp39 and CD40 Ig on Antiphage Ab Production.** The ligand for CD40 (gp39) expressed by activated T cells was recently identified (7, 13). Soluble gp39 (sgp39) is known to bind to CD40 and to provide costimulatory signals to B cells (7). As shown in Fig. 2, sgp39, similar to anti-



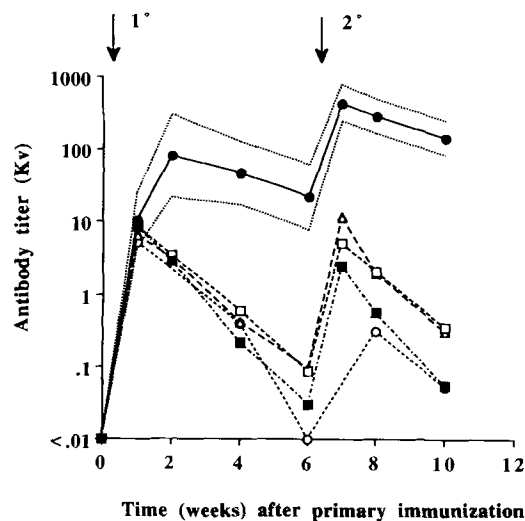
**Figure 2.** Effect of soluble gp39 (sgp39) on the production of phage-specific Ab. Purified B cells obtained from an immunized human volunteer were cultured with various combinations of Ag (phage), IL-10 (10 ng/ml), anti-CD40 (1  $\mu$ g/ml), soluble gp39 (1:4 dilution), or soluble CD72 (1:4 dilution). Mean and 1 SD of triplicate cultures are shown.

CD40, induced antiphage Ab production by B cells costimulated with Ag and IL-10. A control fusion protein, sCD72, had no effect on phage-specific Ab production.

Soluble CD40 Ig has been shown to inhibit the interaction between gp39 (CD40 ligand) expressed on activated T cells and CD40 expressed constitutively on B cells by competitive binding to gp39. To explore the effect of this construct on antiphage Ab production by primed lymphocytes, we cultured PBMC obtained from fully immunized volunteers in the presence of Ag and soluble CD40 Ig. Antiphage Ab production was suppressed in a dose-dependent manner (Fig. 3). In contrast, spontaneous *in vitro* IgG production was not suppressed by the addition of soluble CD40 Ig, indicating that the suppressive effect of soluble CD40 Ig is not caused by a toxic effect on B cells (data not shown). This



**Figure 3.** Effect of CD40 Ig on phage-specific Ab production by PBMC. PBMC ( $2 \times 10^6$ /ml) obtained from an immunized human volunteer were cultured with Ag (phage) and various concentrations of CD40 Ig (4–20  $\mu$ g/ml) or Leu-8 Ig (10  $\mu$ g/ml).



**Figure 4.** In vivo production of phage-neutralizing Ab in normal humans and patients with hyper IgM syndrome. Normal males ( $n = 12$ ) (—●—, geometric mean; .....  $\pm$  SD) and patients with x-linked hyper IgM syndrome and functionally defective gp39 ( $n = 4$ ) (○,  $\Delta$ , □, ■) were immunized with phage twice 6 wk apart (arrows). Mean percent IgG of antiphage Ab in serum collected 2 wk post-secondary immunization was 49% in controls and 0% in all hyper IgM patients.

is further confirmed by the observation that soluble Leu-8 Ig used as control fusion protein had no suppressive effect on phage-specific Ab production. These observations demonstrate that stimulation of CD40 by gp39 plays an important role in specific Ab production *in vitro*.

**Ab Responses to Bacteriophage  $\phi$ X174 in the Hyper IgM Syndrome.** To demonstrate the *in vivo* role of CD40 for specific Ab production, patients with X-linked hyper IgM syndrome due to nonfunctioning gp39 (13) were immunized with bacteriophage  $\phi$ X174. As shown in Fig. 4, all four patients had depressed primary and secondary Ab responses, characterized by lack of amplification and inability to switch from IgM to IgG isotypes. In normal male controls ( $n = 12$ ), 49% of phage neutralizing Ab present in serum 2 wk after secondary immunization is of the IgG isotype. In contrast, none of the four hyper IgM patients had detectable antiphage Ab of the IgG class.

## Discussion

The T cell-dependent Ag, bacteriophage  $\phi$ x174, is a useful tool to study immunologic responses in normal and immunodeficient subjects (8–10, 16). Immunodeficient patients with severe T lymphopenia (subgroup of DiGeorge syndrome, adenosine deaminase deficiency) or with functionally abnormal T cells (subset of common variable immunodeficiency) have a depressed Ab response with defective amplification and class switch, indicating the essential role of T cell help in the synthesis of antiphage Ab *in vivo* (8, 16). *In vitro* synthesis of antiphage Ab by Ag-stimulated PBMC from immunized human subjects has been demonstrated (10). However, purified B cells cultured with Ag produce only minute amounts of

antiphage Ab, suggesting that T cell/macrophage help is required for specific Ab production in vitro (10). In this study we demonstrated that, if anti-CD40 and IL-10 are added to the culture system, Ag-specific Ab production by purified B cells occurs. The amount of phage-specific Ab produced by B cells stimulated with anti-CD40/IL-10 and Ag is comparable to that produced by PBMC cultured in the presence of Ag, suggesting that anti-CD40/IL-10 substitutes the help provided by T cells and/or macrophage in this in vitro system of specific Ab production.

The binding in our in vitro system of anti-CD40 or ggp39 to CD40 initiates signals that will lead to B cell activation; the interaction between gp39 and CD40 in vivo is considered to represent contact-dependent T cell help to B cells (7, 13, 17). The critical role of gp39/CD40 interaction in the induction of Ag-specific Ab production is underscored by our observation that anti-CD40 as well as a soluble form of gp39 induces phage-specific Ab production by purified B cells. Furthermore, CD40 Ig, if added to the culture system, inhibits the production of antiphage Ab, presumably by binding to its ligand, gp39, and thus interfering with the binding of the T cell ligand with its B cell counterpart (7). In addition, our observation that patients with the x-linked hyper IgM syndrome, due to functionally defective gp39 (13), respond to phage immunization with depressed Ab titers, lack of amplification, and failure to switch from IgM to IgG provides further evidence for the important role the gp39/CD40 signaling system plays in the induction of in vivo Ag-specific Ab production. These results indicate that expression of functional gp39 is required for amplification and isotype switching as part of the proper Ag-specific Ab response. Since T cell stimulation via CD3 induces gp39 expression (17), it is likely that interaction of the TCR with Ag-derived peptide presented by APC induces gp39 expression by these activated T cells. It is thus possible that under physiologic conditions induction of gp39 is regulated tightly and limited to Ag-directed cognate T/B cell interaction.

The involvement of IL-10, a potent growth and differenti-

ation factor for human B lymphocytes (6), in Ag-specific Ab production is evident by our observation that B cells cultured with Ag and anti-CD40 produce phage-specific Ab only if IL-10 is added to the system. Consistent with our hypothesis that IL-10 plays an important role in Ag-specific Ab production in vivo is the observation that IL-10 mRNA is upregulated 100-fold in spleen cells during the primary immune response of mice to goat anti-mouse IgD Ab (18). Although IL-4, similar to IL-10, induces proliferation and polyclonal Ig production in anti-CD40-activated B cells (6, 13), IL-4 failed to induce antiphage Ab synthesis in vitro. This failure may be explained by the fact that antiphage Ab consists mostly of IgM and IgG (IgG1, IgG2, and IgG3, but very little, if any, IgG4) isotype (9), and IL-4 induces mainly IgE and IgG4 subclass synthesis (5), while IL-10 effectively causes production of IgM, IgA, and IgG of all four IgG subclasses (6; S. Nonoyama, unpublished observation).

B cells, exposed to a combination of anti-CD40 and IL-10 in the of Ag, secrete large amounts of all Ig isotypes (except IgE), but only minute amounts of phage-specific Ab. However, if Ag is added to the system, antiphage Ab synthesis is greatly increased, indicating that Ag stimulation has a synergistic effect. These results are consistent with previous reports that anti-Ig and anti-CD40 are comitogenic in B cell activation (2), and that B cell activation through crosslinking of surface Ig is enhanced by IL-10 (6). Considering that surface Ig is the only receptor that transmits Ag-specific signals to B cells, we postulate that Ag stimulation provides the initial signal that renders Ag-specific B cells more receptive for the subsequent helper signals by anti-CD40 and IL-10.

The establishment of a B cell culture system producing Ag-specific Ab has allowed us to study molecules involved in activating Ag-primed memory B cells. Ag-specific Ab production by primed B cells depends on three signals: Ag, anti-CD40, and IL-10. In the absence of any one of these reagents, B cells fail to produce specific Ab, suggesting that each component provides a distinct and synergistic signal to B cells.

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