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Research Article

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Biochemical Basis of Synergy between Antigen and T Helper (T_h) Cell-mediated Activation of Resting Human B Cells

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Abstract

We have utilized CD23 expression as a marker for B cell activation in order to investigate the biochemical basis for synergy between antigen and T helper (T_h) cells in the activation of resting human B cells. Our results confirm that while ligation of surface immunoglobulin (sIg) receptors by antigen analogues (e.g., F(ab')₂ goat anti-human IgM) does not lead to CD23 expression, this stimulus markedly enhances CD23 expression induced during antigen specific T_h -B cell interaction or by rIL-4. Utilizing a panel of monoclonal anti-human IgM antibodies, we observed a positive correlation between the capacity of a particular antibody to synergize with rIL-4 in CD23 expression and with B cell growth factor in B cell proliferation; suggesting that synergy in CD23 expression reflects the transduction of a functionally important signal via the sIg receptor. We next assayed analogues of the "second messenger" molecules, released during inositol lipid hydrolysis, for their capacity to amplify CD23 expression. These studies showed that protein kinase C (PKC) activating phorbol esters and the synthetic diacylglycerol analogue, DiC8, synergize with either T_h cells or rIL-4 in CD23 expression, while under no experimental condition does increasing B cell $[Ca^{2+}]_i$ with ionomycin enhance CD23 expression. Taken together, these data suggest that activation of B cell PKC is the crucial biochemical event that primes antigen-activated B cells to respond more vigorously to interaction with T_h cells and/or their soluble products.

Introduction

Antigen binding to surface immunoglobulin (sIg) on resting murine and human B cells stimulates the rapid turnover of membrane inositol lipids (1-3). Inositol lipid hydrolysis results in the formation of two "second messenger" molecules, which link receptor-ligand interactions with cellular responses in a wide variety of cell types: inositol 1,4,5 trisphosphate (IP₃),¹

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1. *Abbreviations used in this paper:* AFC, antibody forming cells; BCGF, B cell growth factor; $[Ca^{2+}]_i$, intracellular free calcium concentration; DG, diacylglycerol; DiC8, dioctanoyl 1,2 diacylglycerol; EN,

which causes a rise in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$), and diacylglycerol (DG) which serves as a cofactor in the activation of protein kinase C (PKC) (4-7). Functionally, antigen-induced B cell activation serves as a proliferative stimulus and readies the B cell to respond to T cell help (8, 9). In contrast to this rather well characterized sequence of antigen-dependent events, neither the biochemical basis of T_h cell induced B cell activation nor the mechanism by which antigen and T_h cells synergize in B cell activation have been elucidated.

In previous studies, we employed cloned, IL-2 dependent, allospecific human T_h cells to investigate the early events in human B cell activation. We reported that within 8-16 hours of major histocompatibility (MHC) specific interaction between allospecific T_h cells and allogeneic B cells, a significant fraction of the B cell pool is induced to express a B cell/monocyte lineage restricted activation antigen, CD23 (10). The unique capacity of the T_h cell-derived lymphokine, IL-4, to trigger CD23 expression, coupled with the requirement for close physical contact between antigen-activated T_h cells and responder B cells for optimal CD23 expression led us to suggest that T_h cells may release IL-4 locally at the site of T_h cell-B cell interaction (11). In this regard, evidence for site directed exocytosis of IL-4 by murine T_h cells has been described (12).

We recently reported that T_h cell-induced CD23 expression by resting human B cells occurs in the absence of enhanced B cell inositol lipid metabolism or a rise in B cell $[Ca^{2+}]_i$ (13). Conversely, ligation of sIg receptors on resting human B cells triggers inositol lipid hydrolysis but does not lead to CD23 expression (13). Of interest, while T_h -B cell interaction does not affect the changes in B cell inositol lipid metabolism or $[Ca^{2+}]_i$ induced by sIg crosslinking (13), Defrance et al. have shown that sIg receptor crosslinking enhances CD23 expression induced by rIL-4 (14). These data suggest that the synergistic effects of antigen and T_h cell signaling on B cell activation may occur at a point(s) distal to B cell inositol lipid hydrolysis and associated "second messenger" generation, and that the CD23 induction assay can be utilized to explore the biochemical basis for this synergy. The experiments described here address this possibility.

Methods

Reagents. rIL-4 was obtained from Genzyme (Boston, MA) and was reconstituted at a concentration of 10,000 U/ml. The monoclonal anti-human IgM antibodies, Mu53, P24, HB57, and 5D7 were generously provided by Dr. David N. Posnett (Cornell University Medical Center, New York, NY), and Dr. Patricia Mongini (The Hospital for

neuraminidase-treated red blood cells; IP₃, inositol 1,4,5-trisphosphate; MFI, mean fluorescence intensity; PDB, phorbol dibutyrate; PKC, protein kinase C; PMA, phorbol 12,13 myristate acetate; sIg, surface immunoglobulin; T_h , T helper cell.

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Joint Diseases, New York, NY). The immunochemical and functional characteristics of these antibodies have been described in detail (15, 16). The mAbs directed against CD23 were generously provided by Dr. Bill Sugden and Dr. Stan Metzenberg (McArdle Institute for Cancer Research, Madison, WI) and the MAb 4F2 was generously provided by Dr. Alice Gottlieb (The Rockefeller University, New York, NY). Phorbol 12,13 myristate acetate (PMA), phorbol dibutyrate (PDB), phorbol, phorbol 13-monoacetate, and dioctanoyl 1,2 diacylglycerol (DiC8) were obtained from Sigma Chemical Co. (St. Louis, MO).

Preparation of target B cell populations. Human peripheral blood or tonsil mononuclear cells were isolated on Ficoll-Hypaque density gradients. T and non-T cells were separated on Ficoll-Hypaque density gradients after rosetting the T cells with neuraminidase-treated sheep red blood cells (EN), as previously described (17). Non-T cells were suspended at 2×10^6 cells/ml in RPMI 1640 (Gibco Laboratories, Grand Island, NY) containing 10% fetal calf serum (Hazelton-Dutchland, Inc., Denver, PA), penicillin and streptomycin 50 U/ml (Gibco Laboratories, Grand Island, NY), 2 mM glutamine (Gibco), "culture medium," and incubated in a horizontally placed plastic tissue culture flask (3024; Falcon Labware, Oxnard, CA) at 37°C, 5% CO₂ for 16 h to remove adherent cells. In some experiments, tonsil nonadherent, non-T cells were enriched for high density (resting) and low density (preactivated) B cell populations on discontinuous Percoll gradients as previously described (18). A typical high density B cell population (Percoll fraction 4, Table I) isolated in this manner contained > 98% sIg, < 1% CD3, < 1% MO₂, 9% 4F2, and 64% sIgD positive cells. The low density B cells (Percoll fraction 1, Table I) were > 98% sIg, < 1% CD3, < 1% MO₂, 47% 4F2, and 46% sIgD positive cells.

T cell clones. The IL-2 dependent human T_h clones employed in these studies have been described previously. Allospecific T_h clones include clone A-7, DR3 reactive (19); clone A-57, DR2 reactive (19); and clone 86, DR1 reactive (20). All of these T_h clones are CD3 positive, CD4 positive, and CD8 negative, and proliferate in a MHC-restricted manner to allogeneic stimulator cells expressing the relevant MHC class II antigen. In addition, both clone A-57 and A-7 have been shown to provide MHC restricted helper activity for allogeneic B cells as measured by the induction of B cell proliferation and differentiation into antibody forming cells (AFC) (19).

CD23 induction assay. The induction of CD23 by cloned T_h cells has been described previously (10). Briefly, 5×10^5 B cells were incubated at 37°C in final medium alone or in the presence of 1.5×10^5 cloned T_h cells. After 16 h, the B cells were assessed for CD23 expression by indirect immunofluorescence staining. As noted in the text, in some studies, culture conditions were varied to allow B cells to interact with different stimuli in a primary and secondary culture period, before assessment of CD23 expression. CD23 antigen was detected using the EBVCS₂ mouse monoclonal antibody (generously donated by Dr. Bill Sugden and Dr. Stan Metzenberg, Madison, WI). Samples were counterstained with fluorescein-conjugated F(ab')₂ fragments of goat anti-mouse IgG (Tago, Inc., Burlingame, CA) and the percentage of positively staining cells determined on a cytofluorograph (Ortho IIs; Ortho Diagnostic Systems, Inc., Westwood, MA), gating on the lymphocyte population. The percentage of B cells was determined by staining with FITC-conjugated F(ab')₂ fragments of polyclonal goat anti-human IgG, IgA, and IgM antibodies. Results are expressed as percentage of the sIg bearing cells positive for CD23 after subtraction of background fluorescence (cells incubated with PBS containing 1% BSA, followed by the fluorescein-goat anti-mouse IgG). Fluorescence intensity was measured on a log scale and is expressed as the mean fluorescence intensity (MFI) of positively staining cells.

Results

Antigen and T_h cells synergize in the induction of CD23 on tonsillar B cells. As noted above, crosslinking of B cell sIg receptors by antigen analogues potentiates the CD23 response induced by rIL-4. To determine if antigen-mediated signaling

also synergizes with T_h-B cell interaction in the CD23 expression assay, purified tonsillar B cells were cultured with medium alone, F(ab')₂ goat anti-human IgM, cloned alloreactive T_h cells specific ("matched") for class II antigens on the B cells surface, or the combination of F(ab')₂ goat anti-human IgM and "matched" T_h cells. A representative study (Fig. 1) confirms our previous observation that sIg crosslinking alone is a poor stimulus for CD23 expression, while matched alloantigen specific T_h cells induce a significant percentage of B cells to express CD23 on their surface. Of interest, costimulation with the antigen analogue, F(ab')₂ goat anti-human IgM, and matched T_h cells significantly increases both the percentage of CD23 positive B cells and the intensity of anti-CD23 staining.

Antibodies to human IgM vary considerably in their capacity to activate B cells, as defined either by the induction of a B cell proliferative response or the enhancement of a proliferative response triggered by T cell derived B cell growth factors (BCGF) (15, 16). For this reason, we studied a panel of monoclonal anti-human IgM antibodies that have been well characterized with respect to affinity, ligand epitope density, domain specificity, and capacity to trigger B cell proliferation in response to BCGF (15, 16). In these studies, we took advantage of the fact that rIL-4 can substitute for antigen specific T_h-B cell interaction in the induction of CD23 expression. Data representative of four different experiments are shown in Fig. 2. Both monoclonal antibodies which have been shown to potentiate B cell proliferation in response to BCGF, HB57 and 5D7, effect synergy in CD23 expression. MAb HB57, which amplifies B cell proliferation to BCGF over a wide range of antibody concentrations, induces the most marked enhance-

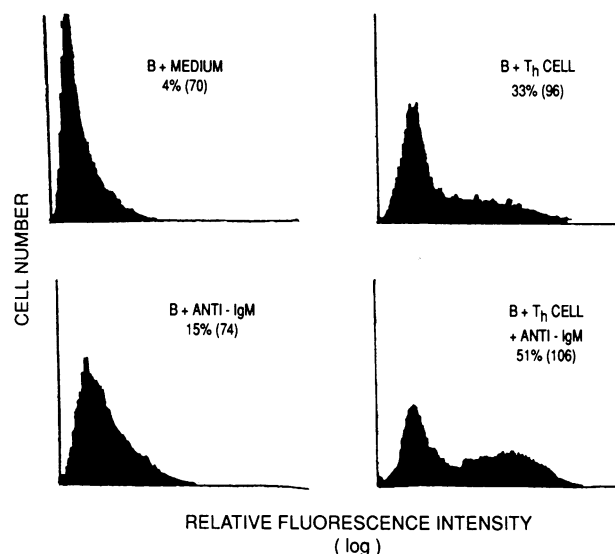


Figure 1. Polyclonal anti-IgM antibodies enhance CD23 expression on human B cells. 5×10^5 tonsillar B cells were cultured with medium, 1×10^5 cloned alloreactive T_h cells specific (matched) for MHC class II antigen on the B cells, 5 μ g/ml of polyclonal F(ab')₂ goat anti-human IgM antibody or both the T_h clone and the polyclonal F(ab')₂ goat anti-human IgM antibody. After 16 h cultures were harvested and B cells assayed for CD23 expression by indirect immunofluorescence staining as detailed in Methods. Data is expressed as percentage of CD23 positive B cells, minus background staining with PBS, and the numbers in parentheses indicate MFI of CD23 expression on positive cells.

ment of CD23 expression. MAb 5D7, which augments proliferation only at high antibody concentrations, is more weakly synergistic. In contrast, antibodies such as Mu53 and P24, which do not enhance B cell proliferation to growth factors, display little synergy in CD23 expression. Taken together, these data suggest that synergy in the CD23 expression assay, between antigen analogues and either rIL-4 or antigen specific T_h -B cell interaction, reflects the delivery of a functionally important signal via the slg receptor.

Phorbol esters can enhance CD23 expression induced during T_h -B cell interaction. The results presented in Figs. 1 and 2 confirm that the CD23 induction assay can be utilized to detect synergy between antigen-mediated signaling and T_h -B cell interaction in human B cell activation. We therefore utilized this assay to investigate the biochemical basis for the synergy observed. To this end, we employed compounds that mimic the activities of "second messenger" molecules released by inositol lipid hydrolysis, the pathway activated by slg cross-linking. The calcium ionophore, ionomycin, was substituted for IP_3 ; and the PKC activator phorbol 12,13 myristate acetate (PMA) for DG. As shown in Fig. 3, B cells incubated for 16 h

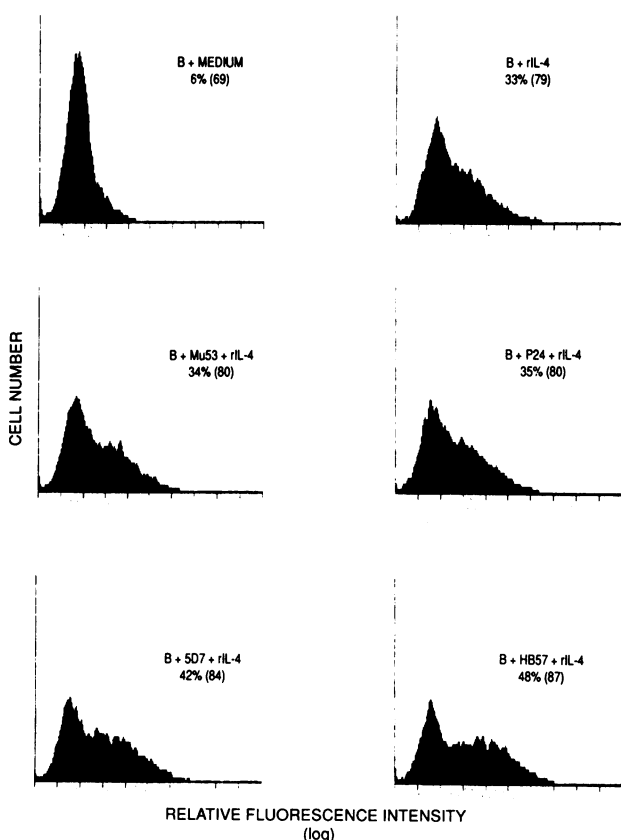


Figure 2. The effect of monoclonal anti-IgM antibodies on IL-4-induced CD23 expression. 5×10^5 tonsillar B cells were cultured with medium alone, rIL-4 (25 U/ml), or the combination of rIL-4 and one of the monoclonal anti-human IgM antibodies indicated; Mu53, P24, 5D7, or HB57. As all anti-human IgM antibodies employed are IgG, CD23 expression was measured by indirect immunofluorescence using the IgM anti-CD23 MAb (EBVCS3) followed by fluorescein-conjugated F(ab')₂ fragments of goat anti-mouse IgM. CD23 expression by B cells cultured with each monoclonal anti-IgM antibody alone was not greater than medium control (data not shown). Data is expressed as indicated in the legend to Fig. 1.

with ionomycin or PMA alone do not express significantly more CD23 than B cells cultured in final medium alone. Similarly, culturing B cells with ionomycin and matched allo-specific T_h cells either has no effect or results in a mild decrease in CD23 expression compared to that induced by matched T_h cells alone. In contrast, co-culture of B cells with matched T_h cells and PMA results in a striking increase in both the mean fluorescence intensity of CD23 staining and the percentage of CD23 positive B cells over that induced by T_h -B cell interaction alone. The marked increase in B cell CD23 expression induced by the combination of PMA and either T_h cells or rIL-4 is a selective event, as the expression of other cell surface activation antigens, including CD25 (Tac), is not upregulated (data not shown).

To determine if T_h cell-induced CD23 expression is restricted to a particular subset of B cells, purified tonsillar B cells were fractionated into low density (activated) and high density (resting) subpopulations by discontinuous Percoll gradient centrifugation. Each subpopulation was cultured with medium alone, an alloreactive T_h clone specific for an MHC class II antigen expressed on the B cell surface (matched) or, as a negative control, an alloreactive T_h clone specific for an MHC class II antigen that the B cells do not express ("mismatched"). In agreement with our previous observations (10), CD23 is induced only during the MHC class II specific inter-

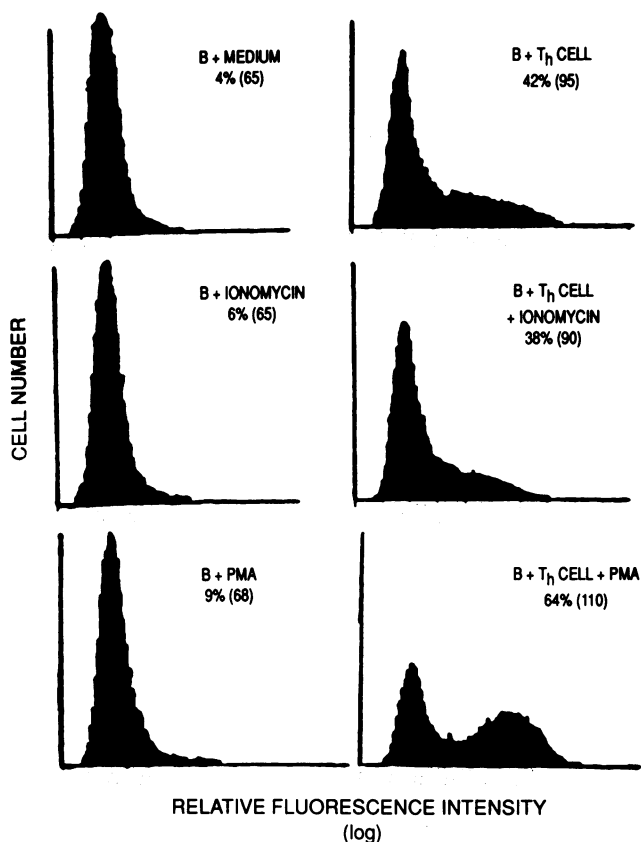


Figure 3. PMA but not ionomycin enhances CD23 expression induced during T_h -B cell interaction. 5×10^5 tonsillar B cells were cultured with medium, ionomycin (0.1 μ M), or PMA (5 ng/ml), in the presence or absence of 1×10^5 MHC class II-matched allo-specific cloned T_h cells. After 16 h cultures were harvested and B cells assayed for CD23 expression by indirect immunofluorescence staining. Data are expressed as indicated in the legend to Fig. 1.

action of cloned T_h cells and responder B cells (Table I). Moreover, B cells responsive in the T_h cell induced CD23 expression assay are enriched in the high-density fractions and depleted in the low density fraction. The paucity of CD23 expressing low density B cells does not simply reflect a shift in kinetics, as similar results were obtained when B cell subsets were assayed 1, 3, 5, and 7 d after MHC specific T_h -B cell interaction (data not shown). Furthermore, poor CD23 expression by low density B cells is not the result of suppression, as the presence of low density B cells does not inhibit T_h cell induced CD23 expression by high density B cells (data not shown). Finally, as shown in Fig. 4, PMA cannot normalize the poor CD23 response of low density B cells, but markedly enhances CD23 expression by the resting B cell pool. Taken together these results suggest that CD23 expression is restricted to resting B cells.

The experiments described above, which involve co-culture of T_h cells and B cells, do not provide information as to the site(s) of action of PMA in CD23 induction. For example, PMA may enhance B cell responsiveness to T_h cell signaling, amplify T_h cell activity, or both. To address this point, we compared the effect of PMA on both T_h cell and rIL-4 mediated CD23 expression. As shown in Table II, PMA enhances CD23 expression in both cultures comprised of allospecific T_h clone cells and resting B cells, and those containing only resting B cells and rIL-4. While these data do not rule out an effect of PMA on T_h cell function, they strongly suggest that the resting B cell is a direct target of PMA action in this system.

Fig. 5 represents a dose-response curve relating CD23 expression to rIL-4 concentration. As shown, B cells cultured with rIL-4 at concentrations below 5 U/ml, express baseline levels of CD23. At higher concentrations of rIL-4, a plateau of CD23 expression is rapidly achieved such that increasing from 50 to 1,000 U/ml of rIL-4 does not significantly enhance either the percent of CD23⁺ B cells or their intensity of staining with anti-CD23 antibody. The addition of PMA to B cells cultured with concentrations of rIL-4 which induce CD23 expression

Table I. Expression of CD23 on Low or High Density B Lymphocytes Cultured with Cloned T_h Cells

Responder B cells	% B cells recovered	% CD23 ⁺ B cells after culture with*		
		Medium	Mismatched T_h Clone	Matched T_h Clone
Unfractionated	—	4	1	36
30/50% (1)	26	1	1	13
50/55% (2)	11	3	<1	50
55/60% (3)	24	2	8	51
60/100% (4)	39	1	1	72

* Unfractionated tonsil B cells, or B cell subpopulations isolated by discontinuous Percoll density gradient centrifugation, were cultured with medium alone, MHC class II matched allospecific cloned T_h cells, or MHC class II mismatched allospecific cloned T_h cells. B cell subpopulations are described with respect to the percentage of Percoll contained within gradient layers on either side of the interface from which the B cells were isolated. Fraction 1 represents the low density B cell population, and fractions 2–4 are increasingly higher density B cell populations. After 16 h the B cells were assayed for CD23 expression by indirect immunofluorescence staining. Data are expressed as percentage of CD23 positive B cells.

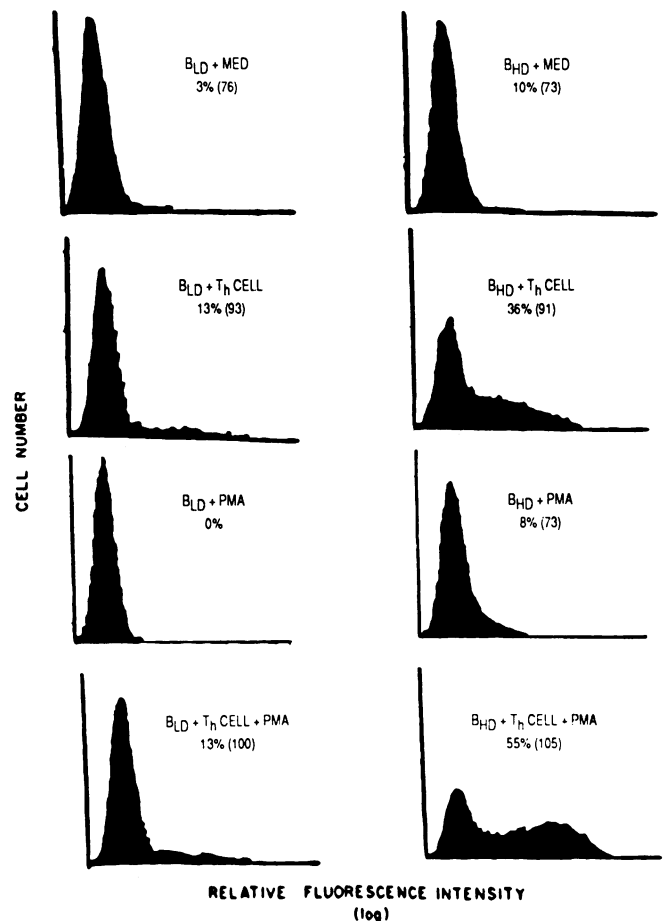


Figure 4. The effect of PMA on T_h cell-induced CD23 expression by high density and low density B cell subpopulations. 5×10^5 high density (B_{HD}) and low density (B_{LD}) tonsillar B cells were cultured with medium alone, "matched" allospecific T_h cells, PMA (5 ng/ml), or the combination of T_h cells plus PMA. After 16 h cultures were harvested and B cells assayed for CD23 expression by indirect immunofluorescence staining. Data is expressed as indicated in the legend to Fig. 1.

results in synergy. Most notable, however, the presence of PMA does not obviate the requirement for a threshold concentration of rIL-4 to initiate detectable CD23 expression.

Increasing B cell $[Ca^{2+}]_i$ partially inhibits CD23 expression. As noted above (Fig. 3) ionomycin decreases CD23 expression induced during T_h -B cell interaction. To determine if this negative effect was due at least in part to increasing B cell $[Ca^{2+}]_i$, we cultured tonsillar B cells with medium alone, rIL-4, or a combination of rIL-4 and PMA and to these cultures added increasing concentrations of ionomycin. As shown in Fig. 6, concentrations of ionomycin above 50 nM proved to be partially inhibitory to rIL-4-induced CD23 expression and this effect could not be overcome by the addition of PMA. Under no experimental condition did the presence of ionomycin augment CD23 expression triggered either by rIL-4 or during T_h -B cell interaction.

Phorbol ester induced synergy in CD23 expression is dependent on PKC activation. Our working hypothesis is that PMA enhances CD23 expression by mimicking the capacity of antigen binding to sIg receptors to activate PKC within the B cell. To investigate this point, we assayed the capacity of a

Table II. PKC Activating Phorbols Selectively Enhance CD23 Expression

Description of culture	CD23 ⁺ B Cells (MFI)	
Experiment 1	Without T _h cells	With T _h cells
B cells + medium	1 (72)	16 (80)
B cells + PMA	6 (78)	25 (92)
B cells + PDB	7 (68)	37 (96)
B cells + P13M	2 (74)	17 (84)
B cells + phorbol	3 (74)	20 (80)
Experiment 2	Without rIL-4	With rIL-4
B cells + medium	1 (72)	32 (84)
B cells + PMA	3 (78)	44 (99)
B cells + PDB	2 (78)	46 (103)
B cells + P13M	5 (78)	17 (82)
B cells + phorbol	1 (75)	23 (87)

Experiment 1: 5×10^5 B cells were cultured with medium alone, PMA (5 ng/ml), PDB (5 ng/ml), P13M (5 ng/ml), or phorbol (5 ng/ml) in the presence or absence of 1×10^5 MHC class II matched allosppecific cloned T_h cells (experiment 1) or in the presence or absence of rIL-4 (experiment 2). After 16 h the B cells were assayed for CD23 expression by indirect immunofluorescence staining. Data are expressed as indicated in the legend to Fig. 1.

panel of phorbol esters, which vary in their ability to activate PKC, to provide a synergistic signal for CD23 expression. As shown, Table II, both PKC activating phorbol esters, PMA and phorbol dibutyrate (PDB), synergize with rIL-4 or direct T_h-B cell interaction to enhance CD23 expression. In contrast, phorbol-13-monoacetate and phorbol, which do not activate PKC, do not effect synergy (Table II).

In additional studies we employed a synthetic rapidly metabolized, membrane permeable, DG analogue, dioctanoyl

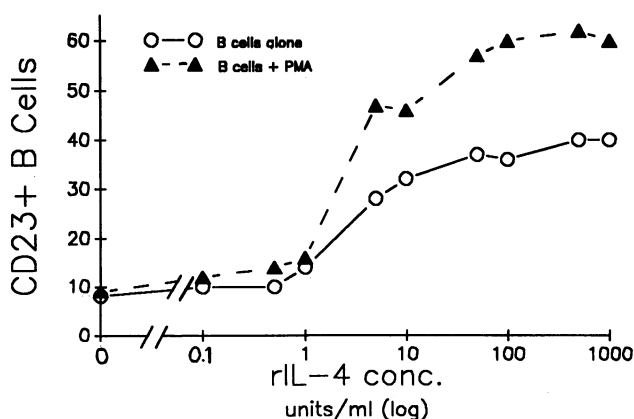


Figure 5. PMA does not replace the requirement for IL-4 as the inducing signal for CD23 expression. 5×10^5 tonsillar B cells were cultured with medium, or with PMA (5 ng/ml), or in the presence of increasing concentrations of rIL-4 (25 U/ml). After 16 h cultures were harvested and B cells assayed for CD23 expression by indirect immunofluorescence staining. The MFI of B cells cultured with concentrations of rIL-4 > 100 U/ml was 83. The MFI of B cells cultured with PMA and concentrations of rIL-4 > 100 U/ml was 98.

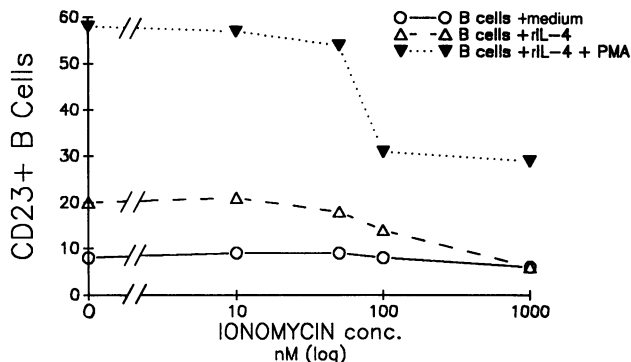


Figure 6. Increasing concentrations of ionomycin partially inhibits CD23 expression. 5×10^5 tonsillar B cells were cultured with medium, rIL-4 (25 U/ml) or rIL-4 plus PMA (5 ng/ml). As indicated these cultures were supplemented with varying concentrations of ionomycin. After 16 h the cultures were harvested and the B cells assayed for CD23 expression by indirect immunofluorescence staining. The MFI of B cells cultured with rIL-4 and < 50 nM of ionomycin was 75 while the MFI of B cells cultured with rIL-4, PMA and < 50 nM of ionomycin was 89. The MFI of B cells cultured in the presence of ionomycin at concentrations < 50 nM was, in all instances, 76.

1,2, glycerol (DiC8), to activate B cell PKC. As depicted in Fig. 7, while the presence of DiC8 alone does not induce CD23 expression, in concert with rIL-4 DiC8 increases both the mean fluorescence intensity of CD23 staining and the percentage of CD23 expressing B cells. Identical results were obtained when allosppecific T_h cells were substituted for rIL-4 as a B cell trigger (data not shown). Taken together, these results strongly suggest that PKC activation in the responding B cell is a pivo-

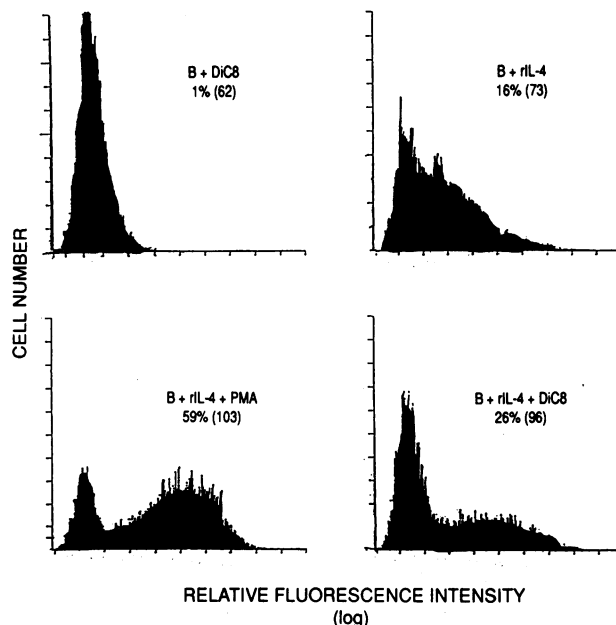


Figure 7. A diacylglycerol analogue, DiC8, provides synergy with rIL-4 in the CD23 expression assay. 5×10^5 tonsillar B cells were cultured with DiC8 (100 μ M), rIL-4 (25 U/ml), rIL-4 and PMA (5 ng/ml), or rIL-4 and DiC8. After 16 h the B cells were assayed for CD23 expression by indirect immunofluorescence staining. Data are expressed as indicated in the legend to Fig. 1.

tal event that allows enhanced CD23 expression in response to rIL-4 or direct T_h-B cell interaction.

Maximal CD23 expression requires the continued presence of both rIL-4 and a PKC activator. During an immune response, antigen specific T_h-B cell interactions are dependent on, and occur subsequent to, antigen binding and processing by the B cell. It is, therefore, logical to postulate that PKC activation, induced by antigen binding, may "prime" the B cell to respond more effectively to a subsequent interaction with T_h cell derived IL-4. Fig. 8 depicts a representative experiment that examines the effect of varying the sequence of B cell interaction with these two classes of stimuli. In this experiment, B cells were precultured for 12 h with medium, the water soluble PKC activating phorbol PDB, or rIL-4. The B cells were then washed, treated as indicated in secondary culture for an additional 16 h, and assayed for CD23 expression. As shown, overnight preincubation with rIL-4 triggers CD23 expression, but this response is not enhanced by subsequent exposure to PDB. In contrast, exposure to PDB during the overnight preculture period primes the B cell to respond more efficiently to rIL-4, as assessed by an increase in the percent of CD23 positive B cells and their intensity of CD23 expression.

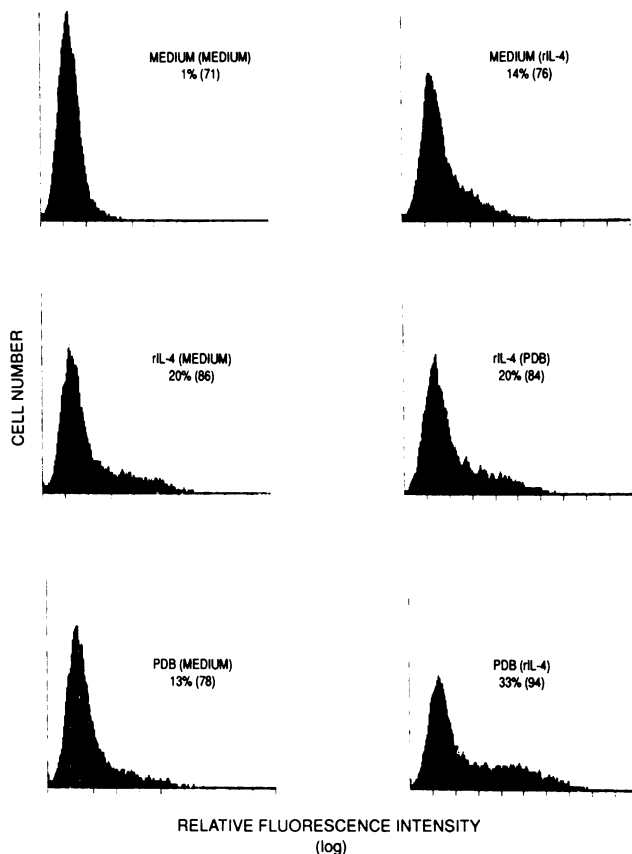


Figure 8. The sequence of B cell stimulation with IL-4 or PDB affects CD23 expression. 5×10^5 tonsillar B cells were placed in primary culture for 12 h, then washed three times and recultured in secondary culture (secondary culture conditions described within parentheses). The final concentration of PDB was 5 ng/ml and rIL-4 25 U/ml. After 16 h in the secondary culture, the B cells were assayed for CD23 expression by indirect immunofluorescence staining. Data is expressed as indicated in the legend to Fig. 1.

Analogous studies involving short term (a few hours) pre-culture provided several important, but essentially negative pieces of data (results not shown). In particular, rIL-4 induced CD23 expression requires prolonged exposure to the lymphokine. Treatment of B cells with rIL-4 for a few hours, followed by washing and overnight culture in medium alone, does not trigger a CD23 response. In addition, short term exposure to a PKC activator, or the combination a PKC activator and rIL-4, does not lead to CD23 expression, nor is it sufficient to enhance CD23 expression triggered by the presence of rIL-4 during secondary culture. We would point out, that in these experiments synergy in CD23 expression is observed if both rIL-4 and a PKC activator are present during the secondary culture period.

Taken together, these studies emphasize the importance, of both the sequence and duration of B cell interaction with both classes of activating stimuli. In particular, they suggest that optimal B cell activation requires prolonged exposure to both a PKC activating signal and IL-4, and that PKC activation may occur before, concomitant with, but not after IL-4 signaling.

Discussion

While it is well established that antigen and T_h cells play crucial roles in the process of B cell activation, the biochemical events which these signals initiate within the B cell are only beginning to be defined. In this report, we have utilized the CD23 expression assay in order to examine the biochemical basis for the synergistic effects of antigen and T_h cell signaling on human B cell activation.

Our results confirm that Ig crosslinking, mediated by the antigen analogues F(ab')₂ goat anti-human IgM and monoclonal murine anti-human IgM antibody, enhances CD23 expression induced by either rIL-4 or antigen specific T_h-B cell interaction. Utilizing a panel of monoclonal anti-human IgM antibodies, well characterized with respect to specificity, affinity, and function, we observed a positive correlation between the capacity of a particular monoclonal antibody to synergize with rIL-4 in the CD23 expression assay and with BCGF in the induction of B cell proliferation (15, 16). Specifically, MAb HB57, which enhances B cell proliferation in response to BCGF over a wide range of antibody concentrations, consistently leads to the most marked synergy in CD23 expression. MAb 5D7, which potentiates B cell proliferation to BCGF only at high antibody concentrations, provides detectable but suboptimal synergy in CD23 expression. In contrast, MAb Mu53 and P24, which do not enhance BCGF-induced B cell proliferation and, have been reported to provide inhibitory signaling for B cell growth (15, 16), are poorly synergistic in the CD23 expression assay. Taken together, these data suggest that the transduction of a functionally important signal via the sIg receptor, leading to enhanced B cell proliferation, may be required for an antigen analogue to induce synergy in CD23 expression.

To investigate the biochemical basis for the synergy observed, we assayed the capacity of analogues of the biochemical second messenger molecules released during inositol lipid hydrolysis to amplify the CD23 response triggered by rIL-4 or during antigen specific T_h-B cell interaction. These studies demonstrate that PKC activating phorbols and the synthetic DG, DiC8, are markedly synergistic. In contrast, under no

experimental condition did increasing B cell $[Ca^{2+}]_i$ with ionomycin result in enhanced CD23 expression. In fact, concentrations of ionomycin greater than 50 nM consistently result in partial inhibition of CD23 expression. Thus, it appears that PKC activation, rather than an increase in B cell $[Ca^{2+}]_i$ is the crucial biochemical event that primes B cells, to respond more vigorously to interaction with T_h cells and/or their soluble products.

The effect of PKC activation on CD23 expression is characterized by both a marked increase in intensity of anti-CD23 staining and in the percentage of high density B cells that express this antigen. Of interest, PKC activation does not enhance the poor CD23 response of low density B cells. These results suggest that PKC activation, presumably mediated under physiologic conditions by the binding of antigen to slg receptors, may increase the fraction of the high density, resting B cell pool, which can respond to T_h cells and their soluble products. Moreover, they are consistent with the fact that activated, low density B cells are relatively refractory to slg-mediated signaling (21).

Our findings also emphasize the importance of both the sequence and duration of B cell interaction with various classes of activating stimuli. In this regard, optimal CD23 expression is induced either by overnight culture of B cells with a PKC activator followed by exposure to rIL-4 alone, or overnight culture of B cells with the combination of a PKC activator and rIL-4. In marked contrast, short term (a few hours) exposure of B cells to rIL-4, PKC activators, or even a combination of the two is not sufficient to trigger B cell activation, as assessed by CD23 expression. Under no experimental conditions does preincubation of B cells with rIL-4 lead to enhanced CD23 expression in response to a subsequent PKC activating signal.

These results imply that maximal expression of CD23 on resting B cells requires substantial activation of the B cell's PKC pool, as well as the sustained contact with IL-4. In addition, synergy in CD23 expression, requires that the activation of B cell PKC occur, or concomitant with, but not after the IL-4 signal is delivered. These findings provide a biochemical basis for the control of B cell activation, and suggest certain constraints on this process. For example, aside from the importance of antigen binding, processing, and presentation by the B cell, as a means of "focusing" antigen specific T cell help, there may be an additional role for the continued presence of antigen to maximally activate the B cell's pool of PKC. In this regard, synergy in CD23 expression requires mitogenic concentrations of PMA 0.5 ng/ml or greater (data not shown). However, we have not directly compared the degree of B cell PKC activation triggered by slg crosslinking versus phorbol esters. Similarly, our results with rIL-4 suggest that prolonged antigen specific T_h -B cell interaction, with local release of lymphokines (e.g., IL-4) is also crucial to optimal B cell activation.

It should be emphasized that the activation of resting B lymphocytes is a complex process that involves both cell division and differentiation into antibody secreting cells. Moreover, the antigen analogues that we have employed in these studies, anti-IgM antibodies, are known to differentially regulate these events; enhancing proliferative responses while profoundly inhibiting differentiation (22, 23). While the relationship between these in vitro effects and the in vivo process of B cell tolerance are speculative, it is of obvious importance to

evaluate the functional potential of human B cells relative to their surface expression of the CD23 molecule. Our data suggest that these studies can be approached using cloned allo-specific T_h cells, which provide MHC restricted "help" for both B cell proliferation and differentiation, in concert with biochemical analogues of the second messenger molecules generated during inositol lipid hydrolysis.

Finally, while the biochemical pathways that T_h cells and IL-4 activate in the B cell have not been defined, it is clear that inositol lipid hydrolysis and increased B cell $[Ca^{2+}]_i$ are not involved (13, 24). Thus, while antigen analogues and rIL-4 act in concert to enhance CD23 expression, these two classes of B cell activators presumably generate distinct sets of "second messenger" molecules in the B cell. As optimal CD23 responses are induced when PKC activation occurs prior to or concomitant with the rIL-4 signal, one explanation for our data is that the PKC dependent phosphorylation of a cellular target of IL-4 enhances the rate of CD23 gene transcription, the stability of CD23 mRNA transcripts, the efficiency of translation, or steps involved in posttranslational modification. The availability of cDNA probes for CD23 should allow this issue to be explored (25).

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