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ACTIVATION OF DENSE HUMAN TONSILAR B CELLS

Induction of *c-myc* Gene Expression via Two Distinct Signal Transduction Pathways[1,2]

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Antibodies to surface Ig or to the B cell marker CD20 trigger resting human B cells in similar yet distinct ways. Either antibody induces five-fold increases in the expression of the protooncogene, *c-myc*, as detected with semi-quantitative Northern blot assays. The induction of *c-myc* mRNA by anti-IgM or anti-CD20 is blocked by inhibitors of protein kinase C (PKC) such as staurosporine and by pretreatment of B cells with phorbol esters to reduce cellular PKC levels. This suggests that PKC is involved in the pathways stimulated by both anti-IgM and anti-CD20. However, anti-CD20, unlike anti-IgM, does not activate significant increases in inositol triphosphate or intracellular-free calcium. Further, anti-CD20-triggered elevation of *c-myc* mRNA is inhibited by pertussis and cholera toxins, whereas the pathway initiated by anti-IgM if anything is stimulated by pertussis toxin and unchanged by cholera toxin. Further differences in the nature of these two signals were seen when the expression of adhesion/recognition molecules were examined. Anti-IgM consistently induces increased expression of the adhesion molecules CD54 (I-CAM-1) and B7/BB-1 on B cells, but anti-CD20 does not. Yet both anti-CD20 and anti-IgM increase class II MHC, CD18 (LFA-1 β-chain) and LFA-3 levels. These data suggest that the way in which B cells are activated may influence their surface phenotype and possibly subsequent migration or cell-cell interactions.

B lymphocytes paradoxically respond in more than one way when stimulated by either cross-linking of their surface IgR or by a linked cell-cell interaction with Ag (1). This apparent paradox has been explained by the finding that surface Ig- and T cell-derived signals, although distinct, are coupled (2). For instance, anti-Ig triggers B cells to enlarge and have increased expression of class II MHC Ag, and these activated B cells are then receptive to T cell-derived signals such as IL-4 (3-5). In other words, a sequential two or three step model of B cell activation can be evoked to explain why B cells respond to more than one signal (6).

Not all data are consistent with a single stepwise pathway for B cell activation; for instance, LPS apparently triggers resting B lymphocytes differently than anti-Ig (4). Whereas IL-4, can also activate resting B cells to enlarge and express increased levels of class II Ag (3) other agents such as mAb to certain B cell-associated surface molecules such as CD20, like anti-Ig, can also trigger B cells to enter the cell cycle (7, 8). Thus, the possibility exists that resting B cells can be activated physiologically via more than one distinct pathway.

In order to address this possibility, we have compared the ability of anti-Ig antibodies vs antibodies to CD20 to activate the early competence gene, *c-myc*, in a resting relatively homogeneous human B cell population. We selected CD20 signaling for a detailed comparison because anti-CD20 like anti-Ig can induce increased mRNA expression of the *c-myc* gene (9) and can stimulate increases in class II protein levels and entry of B cells into the cell cycle (7, 10); however, unlike anti-Ig, antibodies to CD20 do not increase the levels of intracytoplasmic-free calcium in B cells (11, 12). Furthermore, recently we (13) and others (14, 15) have isolated cDNA encoding CD20. The predicted protein has multiple transmembrane segments and has both its N-terminus and C-terminus in the cytoplasm. The structure of CD20 is unlike any other lymphocyte-associated molecule reported to date, suggesting it may mediate a unique function. Here we show that early signal events initiated via CD20 or surface Ig are clearly distinct.

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[2] Address correspondence and reprint requests to Dr. Michael W. White, Veterinary Molecular Biology, Marsh Labs, Montana State University, Bozeman, MT 59717.
[3] Abbreviations used in this paper: W7, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride; PKC, protein kinase C; HA1004, N-(2-guanidinoethyl)-5-isoguinolinesulfonamide hydrochloride; Ins-P, inositol phosphate.

MATERIALS AND METHODS

Materials. Trifluorperazine dihydrochloride, W7, and forskolin were purchased from Sigma. Ionomycin and PMA were from Calbiochem. HA1004 was purchased from Seikagaku America (St. Petersburg, FL). Staurosporine was obtained from Kamiya Biomedical Co. (Thousand Oaks, CA). Cholera and pertussis toxins were purchased from LIST Biological (Campbell, CA).

Cell culture and antibodies. Dense tonsilar B lymphocytes and B cell lines were prepared and cultured as previously described (16). The antibodies 1F5 (IgG2a), 2C3 (IgG1), and LB2 (IgG2b) specific for

CD20, IgM, and CD54 (I-CAM-1), respectively, have been described (16–18). Other antibodies used in these studies were, BB1 anti-B7/BB1 (17); HB10a anti-DR class II (17); G28-1 (IgG1) specific for CD37 (19); 60.3 anti-CD18 (LFA-1 β-chain) kindly provided by Dr. John Hansen (Harvard Medical School, Boston, MA) (20); TS/219 anti-LFA-3 (21) kindly provided by Dr. Tim Springer (Fred Hutchinson Cancer Research Center, Seattle, WA); δ-TA4-1 (IgG1, American Type Culture Collection HB-70, Rockville, MD) specific for IgD; and polyclonal $F(ab')_2$ goat anti-human IgM (The Jackson Laboratories Bar Harbor, ME). The conjugation of 2C3 to Sepharose 4B beads and the generation of 1F5 Fab fragments followed previously described methods (16). Staining and quantification of cell surface CD20 or IgM was performed using fluorescien-conjugated antibodies on a modified FACS IV cell sorter (Becton Dickinson, Mountain View, CA) as previously described (16, 17).

mRNA purification and analysis. Resting cells were activated with the following antibody quantities: 10 μg/ml of intact 1F5; 10 μg/ml of 1F5 Fab fragments; 20 μg/ml of 2C3 or 2C3-Sepharose 4B; 10 μg/ml of G28-1; 5 μg/ml of δ-TA4-1. A low m.w. human B cell growth factor from Cellular Products (Buffalo, NY) was used at a final concentration of 5% (v/v). rIL-1 was kindly provided by Dr. Steve Dower (Immunex Corp., Seattle) and used at 10 U/ml/10^6 cells. Total cellular RNA was purified by guanidine/phenol extraction (22). RNA was separated by electrophoresis on formaldehyde/agarose gels and blotted onto nitrocellulose filters (23). The blots were hybridized and *c-myc* mRNA levels determined using [^{32}P]-labeled nick-translated plasmid insert as previously described (24). Equal amounts of RNA were loaded per gel lane in each experiment and the loadings checked by reprobing each blot for glyceraldehyde-3-phosphodehydrogenase message. The *c-myc*-containing plasmid was derived by subcloning a mouse 350-bp *Pst*-1 genomic fragment containing most of exon 2 (25). The glyceraldehyde-3-phosphodehydrogenase probe was a chicken cDNA *Pst*-1 fragment subcloned into plasmid vector pGEM-2 (26). Autoradiographs exposed within the linear range of the film were quantified by densitometric scanning.

Intracellular calcium determinations. For the calcium measurements, cells were loaded with acetoxymethyl ester of Indo-1 (Molecular Probes, Junction City, OR) and the analysis performed by flow cytometry as previously described (11).

Ins-P measurements. Dense tonsilar B cells (10^7/ml) were incubated for 18 h at 37°C in inositol-free medium containing 5 mg/ml added inositol and 20 μCi of [^{3}H]-inositol per 10^7 cells. Viable dense cells were washed and resuspended in RPMI 1640 containing 35 mg/ml inositol. The Burkitt's lymphoma line, Ramos, was labeled for 4 days with medium containing 2 μCi of [^{3}H]-inositol per ml. Experimental treatments were performed at 37°C on cells suspended at 3×10^7 (Ramos) or 1.5×10^8 (tonsil) cells per ml, using 5 μg/10^6 cells anti-IgM (polyclonal $F(ab')_2$ goat anti-human IgM) or 2 μg/10^6 cells 1F5. Experiments were terminated by the addition of 0.3-ml aliquots of cell suspension to 0.5 ml of cold TCA with added phytate (sodium hexaphosphate, Sigma Chemical Co., St. Louis, MO; 250 μg/ml) and phytate hydrolysate (25 μg/ml) (27). Cell lysates were spun and the pellet was washed with TCA. The combined supernatants were washed five times with 2 ml of hydrated ether. Ether was allowed to evaporate from the final aqueous phase, to which 0.1 ml of 50 mM EDTA was added. Samples were stored at −20°C for later analysis by HPLC.

HPLC analysis. Aqueous cell extracts were analyzed using a Partisphere 5 SAX column (Waters Corp., Milford, MA). Eluted [^{3}H]-labeled compounds were identified by comparison with elution times of authentic titrated standards from Amersham Corp. (Arlington Heights, IL) (Ins-1-P, -1,4,P_2, -1,4,5-P_3, and -1,3,4,5-P_4) or New England Nuclear (Boston, MA) (Ins-1,3,4-P_3). The elution gradient system was composed of ultrapure water and 2.0 M ammonium phosphate pH 3.8. The gradient commenced as pure water advancing to 0.05 M ammonium phosphate over 25 min. Ammonium phosphate was then raised to 0.2 M and held for 10 min, then increased from 0.54 to 0.56 M over 20 min, and then held at 0.8, 1.6, and 2.0 M for 15, 10, and 15 min, respectively. Half-min fractions were collected and counted by liquid scintillation counter with correction for quench. Peaks were expressed as a fraction $\times 10^5$ of the total number of counts eluted from the column. We have found that this value is large and is unaffected by changes in the level of total Ins-P that might result from experimental treatment. This normalization controls for variation between samples in the extraction procedure.

Ins-P analysis are expensive and labor intensive, so that it is not always possible for all experiments to contain all the desired treatments. Between-experiment variation is usually in the form of differences in absolute levels of ^{3}H-inositol in both cell types. The difference is most marked in tonsilar cells, which in addition load and metabolize ^{3}H-inositol poorly yielding low levels of isotope for analysis. Three separate experiments were performed with dense tonsilar cells and with the Ramos cells we have performed experiments four times (anti-IgM treatment) or once (anti-CD20 treatment).

Expression of adhesion/recognition molecules. Blood E rosette negative and leucine methyl ester-treated B cells were activated for 48 h with anti-IgM or anti-CD20 antibodies as described in the mRNA expression studies above. Cells were then stained with fluorescein labeled mAb to CD40 (28) and with one of the following R-phycoerythrin-conjugated mAb: 60.3 anti-CD18 (LFA-1 β-chain)(20); LB-2 anti-CD54 (I-CAM-1)(17); BB1 anti-B7/BB1 (17, 29); or HB10a anti-DR class II (17). Cell preparations contained >80% B cells as detected on a FACScan analyzer (Becton Dickinson). Two color analysis were performed as described (17) with a FACScan set on a 60 channel log scale, where a four channel increase represents approximately a doubling of fluorescence.

RESULTS

Induction of c-myc mRNA levels by anti-CD20. We compared the ability of an anti-IgM mAb, 2C3, and the anti-CD20 mAb, 1F5, to induce increases in the level of *c-myc* mRNA in resting tonsilar B cells. The kinetics of *c-myc* mRNA induction by anti-IgM were slower in the dense tonsil B cells than had been shown previously for the B lymphoma cell line WHEI 231 (30). The peak induction of *c-myc* mRNA by either 1F5 or 2C3 was 4 to 6 h after antibody addition (data not shown) and 4 h was chosen for the following studies. From cells treated for 4 h with the antibodies, total cellular RNA was purified and analyzed on Northern blots as described (24). Representative results are presented in Figure 1. The addition of anti-CD20 was sufficient to increase the level of *c-myc* mRNA fivefold (Fig. 1*A*, *lane 2*). The combination of 1F5 and BCGF did not induce *c-myc* mRNA levels over that seen with 1F5 alone. Fab fragments of 1F5 did not increase the level of *c-myc* mRNA indicating that cross-linking of the CD20 molecule is required (Fig. 1*A*, *lane 1*). These results are consistent with earlier studies showing the induction, by anti-CD20 (1F5), of *c-myc* mRNA in peripheral blood B cells (9). Simple binding of a pan B cell-specific mAb such as G28-1 (to CD37 which is expressed at similar levels as CD20) was insufficient to trigger *c-myc* expression (Fig. 1*B*, *lane 7*).

Unlike 1F5, the addition of whole anti-IgM mAb, 2C3,

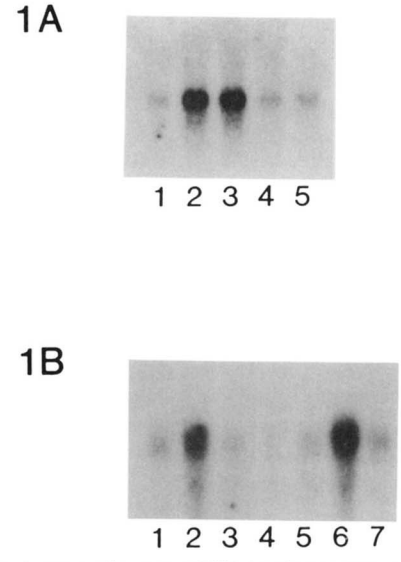

Figure 1. Induction of c-myc mRNA levels in resting B lymphocytes. Resting B cells were stimulated for 4 h with several mAb and lymphokins. RNA was then isolated and Northern analysis performed as described in *Materials and Methods*. *A*, *lane 1*, FAB fragments of mAb 1F5 (anti-CD20); *lane 2*, 1F5 intact; *lane 3*, 1F5 and BCGF; *lane 4*, BCGF; *lane 5*, no addition control. *B*, *lanes 1* and *4*, no addition control; *lane 2*, δ-TA-4 (anti-IgD); *lane 3*, IL-1; *lane 5*, intact mAb 2C3 (anti-IgM); *lane 6*, 2C3-Sepharose 4B; *lane 7*, mAb G28-1 (anti-CD37).

did not result in elevated *c-myc* mRNA (Fig. 1*B*, *lane 5*). The level of *c-myc* mRNA was increased, however, if the 2C3 antibody was first conjugated to Sepharose 4B beads (Fig. 1*B*, *lane 6*) or if a goat $F(ab')_2$ anti-IgM antiserum was used (data not shown). The magnitude of the elevation in *c-myc* mRNA induced by 2C3-Sepharose 4B was identical and had similar kinetics to the increase induced by anti-CD20 (data not shown). It is likely that free 2C3 anti-IgM antibody cannot elevate *c-myc* if its Fc domain is available for binding to B cell FcR because it is well known that cross-linking cell surface IgM and the FcR generates a growth inhibitory signal (31, 32). In subsequent experiments either 2C3 mAb linked to Sepharose beads or $F(ab')_2$ anti-μ heteroanti-sera were used to avoid Fc-associated effects.

Early membrane signals induced by anti-CD20. Increases in cytosolic-free calcium and Ins-P_3 have been demonstrated to occur within minutes after treating resting B-cells with anti-Ig antibodies (33, 34). Inasmuch as either anti-CD20 or anti-IgM antibodies can cause an elevation in *c-myc* mRNA, we examined whether anti-CD20 might have a similar effect on these early events. Calcium levels were measured by cell sorter using the fluorescent indicator, Indo-1. In agreement with previous reports, the addition of anti-IgM stimulated an immediate rise in intracellular calcium. Within 3 min the intracellular calcium concentration increased fourfold from 137 nM in untreated cells, to over 400 nM. In contrast to anti-IgM, the addition of the anti-CD20 resulted in no detectable increase in intracellular calcium during the 10-min time course of the experiment. This experiment was repeated five times using different lots of anti-CD20 antibody with and without cross-linking the anti-CD20 mAb with rat anti-mouse κ-chain second step mAb, 187.1 (11) and the time course was extended to 60 min; however, no elevation in intracellular calcium was observed in any experiment.

Increases in cellular Ins-P_3 levels have been shown to occur after cross-linking the IgR (34, 35). Using HPLC analysis (see *Materials and Methods*) we examined whether the addition of anti-CD20 to resting B cells would also cause an increase in cellular Ins-P_3. As expected, the stimulation of the Ramos Burkitt's lymphoma line (Fig. 2) or dense tonsilar B cells (Table I) with the polyclonal Ab to surface IgM resulted in a rapid increase in the level of Ins-1,4,5-P_3. The initial rise in Ins-1,4,5-P_3 is followed closely by a more sustained elevation of Ins-P_4 and by a delayed accumulation of Ins-1,3,4-P_3 and Ins-1,4-P_2 in keeping with the expected path of metabolism of the Ins-1,4,5-P_3 messenger (36). Even allowing for the difference in degree of labeling between the two cell types, the response in the Ramos line is more pronounced than the tonsilar cell response, both following a qualitatively similar pattern. The difference in the effect between the two types of cell is probably due to the corresponding difference in their expression of surface IgM (37). The time course of the rise and fall of the Ins-1,4,5-P_3 level in both cell types agrees well with the nature of the calcium release elicited by Ab to surface IgM (Fig. 2).

In the Ramos cells, stimulation with anti-CD20-induced slight changes in the levels of Ins-1,4,5-P_3 and InsP_4 and a fivefold increase in Ins-1,3,4-P_3 (Fig. 2). These increases were small compared to anti-IgM stimulation, which induced 4-fold increases in Ins-1,4,5-P_3 and Ins-P_4 and a 50-fold increase in Ins-1,3,4-P_3. Tonsilar cells treated with anti-CD20 also showed signs of inositol phospholipid metabolism (Table I) although the increases in inositol phosphate levels were much less substantial than those induced by anti-IgM stimulus. Anti-CD20 treatment failed to elicit the sustained Ins-P_4 release and the dramatic accumulation of Ins-1,3,4-P_3 that follow cross-linking by anti-IgM.

To control for non-specific effects of mAb binding, we tested the inositol phosphate levels in Ramos cells treated with G28.1 (19) an anti-CD37 mAb. None of the inositol phosphates measurable with our assay exceeded control levels for the duration of a 10-min stimulation with anti-CD37. We thought possible that PKC might have an effect on the metabolism of inositol phosphates distinct from the direct results of Ins-1,4,5-P_3 release. In an attempt to mimic such an effect, we used PMA as an agonist. After 15 min in the presence of 10 ng/ml PMA, the level of Ins-1,4,5-P_3 was raised to twice the control level. This result was obtained in two separate experiments. PMA treatment was followed for 60 min, during which time the only other inositol phosphate isomer to exhibit a significant elevation was Ins-P_4, 35% above control at 60 min. Figure 3 compares Ins-1,4,5-P_3 levels in Ramos cells treated with anti-CD20, anti-CD37, of PMA. Our data indicate that the effect of anti-CD20 on inositol phosphate metabolism is specific and may include a component of change due to the action of PKC.

Involvement of PKC in increase in c-myc mRNA induced by anti-CD20 or anti-IgM. The differences observed in the early signals generated by cross-linking either surface IgM or CD20 suggested the possibility that these stimuli may use distinct pathways to elevate *c-myc* message. Several pharmacologic agents were examined for their effect on the induction of *c-myc* mRNA by the anti-CD20 or anti-IgM mAb; the results from these experiments are summarized in Table II. In all of these experiments the compounds were added at the time of stimulation and the cells harvested after 4 h of treatment.

Trifluorperazine, a phenothiazine derivative known to inhibit equally PKC and calmodulin-dependent kinases (38) strongly inhibited the induction of *c-myc* mRNA by either antibody suggesting the involvement of PKC and/or calmodulin-dependent kinases in the *c-myc* induction pathways. A similar result was observed using the antibiotic staurosporine. Staurosporine is the most potent inhibitor of PKC yet identified with a K_i of 0.7 nM. The addition of staurosporine at 0.1 and 1.0 μM effectively blocked the induction of *c-myc* mRNA by either anti-CD20 or anti-IgM. Inasmuch as staurosporine is also known to inhibit cAMP and cGMP kinases, we examined the effect on *c-myc* mRNA induction of the isoquinolinesulfonamide, HA1004. HA1004 is a potent inhibitor of the cyclic nucleotide-dependent kinases and only weakly inhibits PKC (39). Concentrations of HA1004 up to 30 μM were ineffective in blocking the elevation of *c-myc* mRNA stimulated by these antibodies suggesting that the cyclic nucleotide-dependent kinases are not required for *c-myc* induction by either anti-IgM or anti-CD20.

The naphthalenesulfonamide, W7, has been reported to inhibit PKC and calmodulin-dependent kinase catalyzed reactions with K_i of 110 and 6 μM, respectively (40, 41). At 10 μM W7 the induction of *c-myc* mRNA by either antibody was relatively unaffected whereas 40 μM W7

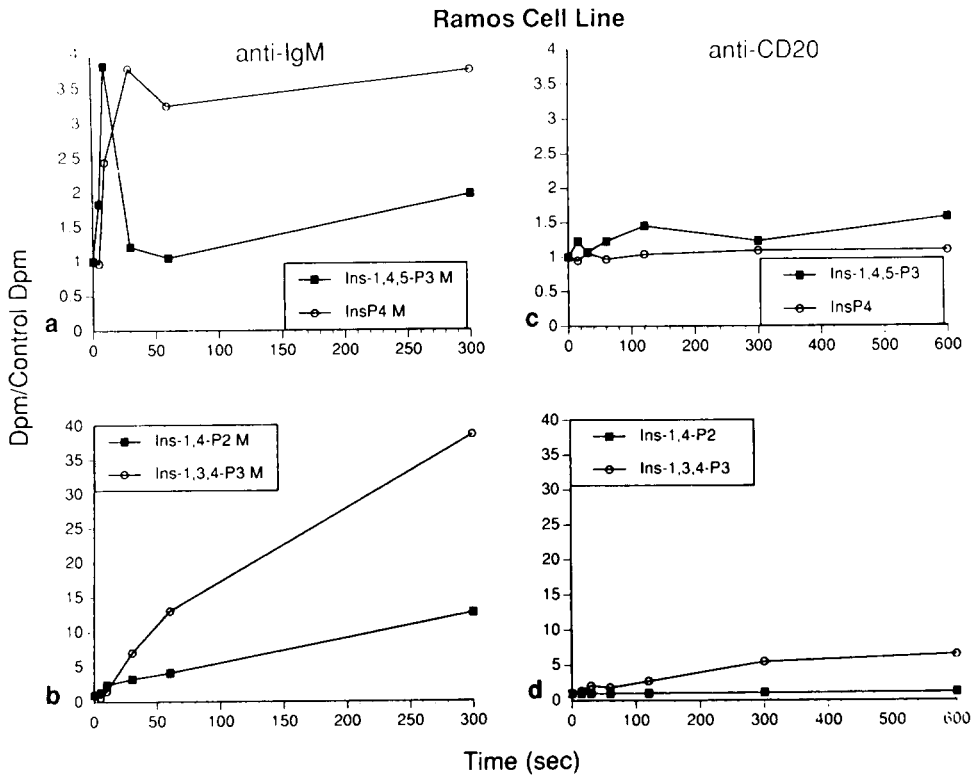

Figure 2. Inositol phosphate levels in Ramos cells after anti-IgM and anti-CD20 stimulus. Ramos cells were grown with [^{3}H]-inositol, treated with anti-IgM or anti-CD20 for various times and analysed for levels of inositol phosphates as described in *Materials and Methods*. *A* and *B*, anti-IgM, control levels of radioactivity in cpm were Ins-1,4,5-P_3, 102; $InsP_4$, 54; Ins-1,3,4-P_3, 23; Ins-1,4-P_2, 213. *C* and *D*, anti-CD20, control levels of radioactivity in cpm for Ins-1,4,5-P_3, 123; $InsP_4$, 462; Ins-1,3,4-P_3, 52; Ins-1,4-P_2, 2231.

TABLE I

Ins-P levels in dense tonsillar cells

Time (s) Stimulus	0		15	60		300		600	
			Anti-IgM	Anti-IgM	Anti-CD20	Anti-IgM	Anti-CD20	Anti-IgM	Anti-CD20
	cpm[a]								
Expt. 1									
Ins-1,4-P_2	408	17.77	17.07	18.20	17.83				
Ins-1,3,4-P_3	0	0	0	1.14	0				
Ins-1,4,5-P_3	225	9.79	13.18	10.25	9.89				
Ins-P_4	93	4.04	9.02	13.99	4.02				
Expt. 2									
Ins-1,4-P_2	156	5.59				8.14	6.84		
Ins-1,3,4-P_3	0	0				5.59	0.75		
Ins-1,4,5-P_3	161	5.77				7.09	5.64		
Ins-P_4	66	2.38				10.68	4.45		
Expt. 3									
Ins-1,4-P_2	272	4.01					5.20	5.79	6.01
Ins-1,3,4-P_3	8	0.19					0.72	7.30	0.15
Ins-1,4,5-P_4	140	2.40					3.22	4.09	3.35
$InsP_4$	155	2.67					2.99	9.16	3.01

[a] Values are cpm above background for each isomer. All other values are normalized dpm (see *Materials and Methods*).

inhibited *c-myc* mRNA accumulation by 80%. A similar dose response was seen in phorbol ester-activated cells. The *c-myc* mRNA levels in PMA activated-cells treated with 10 μM W7 were 75% of control; whereas 40 μM W7 inhibited the induction by 50%. The above results with anti-CD20, anti-IgM, and PMA suggest that the inhibition of *c-myc* induction by W7 involves a block to PKC activity not an inhibition of a calmodulin-dependent kinase.

The results from the inhibitor studies strongly suggest that activation of PKC is required for the induction of *c-myc* message by anti-CD20 or anti-IgM. To test this hypothesis further, we examined the inducibility of *c-myc* mRNA in B cells after PKC down-regulation with phorbol ester. Resting cells were treated for 40 h with 100 nM PMA, a strategy that has previously been demonstrated to dramatically reduce PKC activity from a variety of cell types (42, 43). The results from this experiment (Fig. 4) show that the induction of *c-myc* mRNA levels by either antibody is blocked by phorbol ester pretreatment. The loss of inducibility of *c-myc* mRNA, in the phorbol ester-treated cells, was not due to a reduction in either IgM or CD20 cell surface proteins as measured by fluorescent cell sorter (data not shown). Once again the results are consistent with the involvement of PKC in the signaling pathways triggered by cross-linking cell surface IgM or CD20.

Affect of cholera and pertussis toxins on accumulation of c-myc mRNA. Cholera toxin has been reported to prevent the proliferation of anti-IgM-stimulated mouse B lymphocytes. This does not appear to be due to the inhibition of early signals such as increases in intracellular calcium or Ins-P_3 (4). We have explored whether cholera or pertussis toxins, added in combination with anti-CD20 or anti-IgM, effect the induction of *c-myc* mRNA. Total

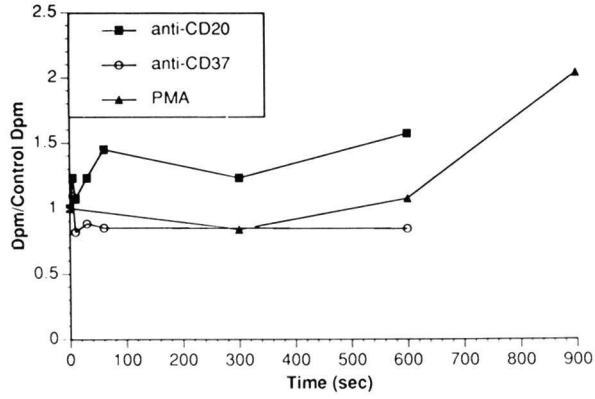

Figure 3. Inositol-1,4,5-trisphosphate levels in tonsillar cells after anti-CD20, anti-CD37, or PMA stimulus. Ramos cells were grown with ^{3}H-inositol, treated with anti-IgM, anti-CD20, or PMA for various times and analyzed for levels of inositol phosphates, as described in *Materials and Methods*. Control levels of radioactivity in cpm for anti-CD20, anti-CD37, and PMA, respectively, were: 123, 60, and 79.

TABLE II
Inhibition of c-myc mRNA levels

Inhibitors	Percent of Control	
	Anti-CD20	Anti-IgM
Control (no addition)	100	100
30 μM Trifluorperazine	5	4
10 μM W7	98	86
40 μM W7	20	21
0.1 μM Staurosporin	3	4
1.0 μM Staurosporin	<1	<1
7.5 μM HA1004	98	101
30 μM HA1004	100	106

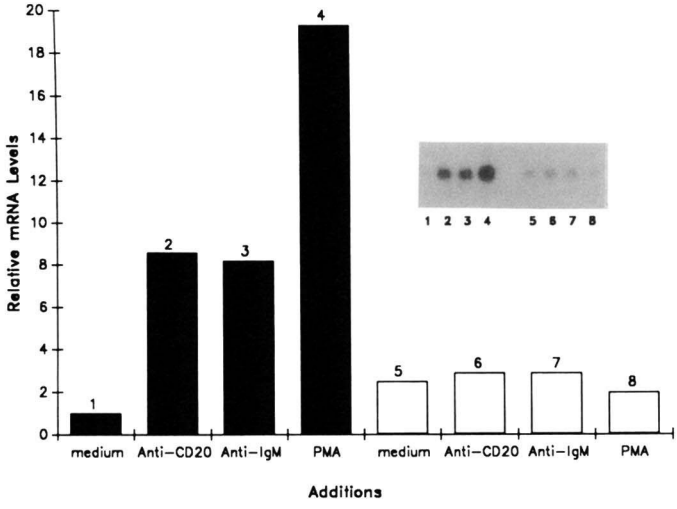

Figure 4. The effect of depleting PKC on the induction of c-myc mRNA by anti-IgM or anti-CD20. Resting B cells were pretreated with the phorbol ester, PMA (100 nM), for 40 h. The cells were then washed with PBS and activated with either 30 nM PMA or mAb 1F5 (anti-CD20) or 2C3-Sepharose 4B (anti-IgM). RNA was isolated from 4 h-activated cells, analyzed by Northern blot, and compared to parallel controls that were not exposed to 100 nM PMA pretreatment. *Closed bars*, no PMA pretreatment; *open bars*, 40 h PMA pretreatment. The number designations (1 to 8) above each *bar* correspond to the lane designations in the inset autoradiograph.

RNA was prepared from cells treated for 4 h and *c-myc* mRNA levels determined by Northern analysis. The results from this experiment are summarized in Table III. Cholera toxin at 0.1 or 10 ng/ml had little effect on the elevation of *c-myc* mRNA levels induced by anti-IgM. In contrast, 10 ng/ml cholera toxin inhibited the anti-CD20 induction of *c-myc* mRNA levels by nearly 70%. The inhibition of anti-CD20 signaling does not appear to be

TABLE III
Effect of cholera and pertussis toxins on c-myc mRNA levels

Additions	Percent of Control	
	Anti-CD20	Anti-IgM
Control (no addition)	100	100
0.1 ng/ml cholera toxin	100	142
10 ng/ml cholera toxin	34	90
10 ng/ml pertussis toxin	103	265
50 ng/ml pertussis toxin	66	149
30 mM forskolin	101	98
90 mM forskolin	102	100

an indirect result of cholera-induced cAMP levels because 90 mM forskolin, which causes a fivefold increase in cAMP in these cells (data not shown), had no effect on the ability of either antibody to increase *c-myc* mRNA. Similar differences between anti-IgM and anti-CD20 were evident when pertussis toxin was used; pertussis toxin enhanced the induction of *c-myc* mRNA caused by the anti-IgM antibody whereas 50 ng/ml pertussis toxin inhibited the normal *c-myc* mRNA increase stimulated by anti-CD20. The inhibitory or stimulatory effect of these toxins appear to be acting at a step prior to the activation of PKC, since the induction of *c-myc* mRNA by PMA in these cells is unaffected by either toxin (data not shown).

Cross-linking surface IgM vs CD20 has distinct effects on expression of adhesion/recognition molecules. Although it is clear that signaling via surface IgM is different from signaling via CD20, it is not clear why resting B cells require more than one means to be activated. One possibility is that the way B cells are initially activated affects expression of adhesion molecules and perhaps then their migration or interactions with other cells. To assess this possibility, we activated resting tonsilar B cells with either anti-IgM of anti-CD20 antibodies and measured the expression of several adhesion/recognition molecules (Fig. 5) including CD18 (LFA-1 β-chain) and one of its ligands CD54 (I-CAM-1) (43); B7/BB1, a ligand for CD28 (29); and class II that interacts with CD4 (44). CD18 expression was increased by both anti-IgM and anti-CD20 (Fig. 5) with CD20 mAb consistently being a better stimulus than anti-IgM. In contrast, although anti-IgM could induce increases in CD54 and B7/BB1 (Fig. 5) (45), anti-CD20 stimulation decreased expression of these molecules. As noted before (10, 46), both anti-IgM and anti-CD20 also induced increases in class II MHC on B cells although the induction of class II was not as dramatic with the tonsilar B cells as we have seen with less activated blood B cells. These results support the concept that the way B cells are stimulated affects which cell adhesion/recognition molecules are elevated.

DISCUSSION

In this study we have shown that small dense human B lymphocytes can be rapidly activated by one of two clearly distinct signaling pathways initiated by cross-linking of either surface IgM or surface CD20. Whereas anti-IgM induced active metabolism of inositol phospholipids, as measured by large increases in the formation of inositol phosphates including the calcium mobilizing Ins-1,4,5-P_3, anti-CD20 does not elevate the level of intracellular calcium and only weakly affects inositol phospholipid metabolism. The surface-IgM or CD20 signaling pathways also differ in their response to cholera and

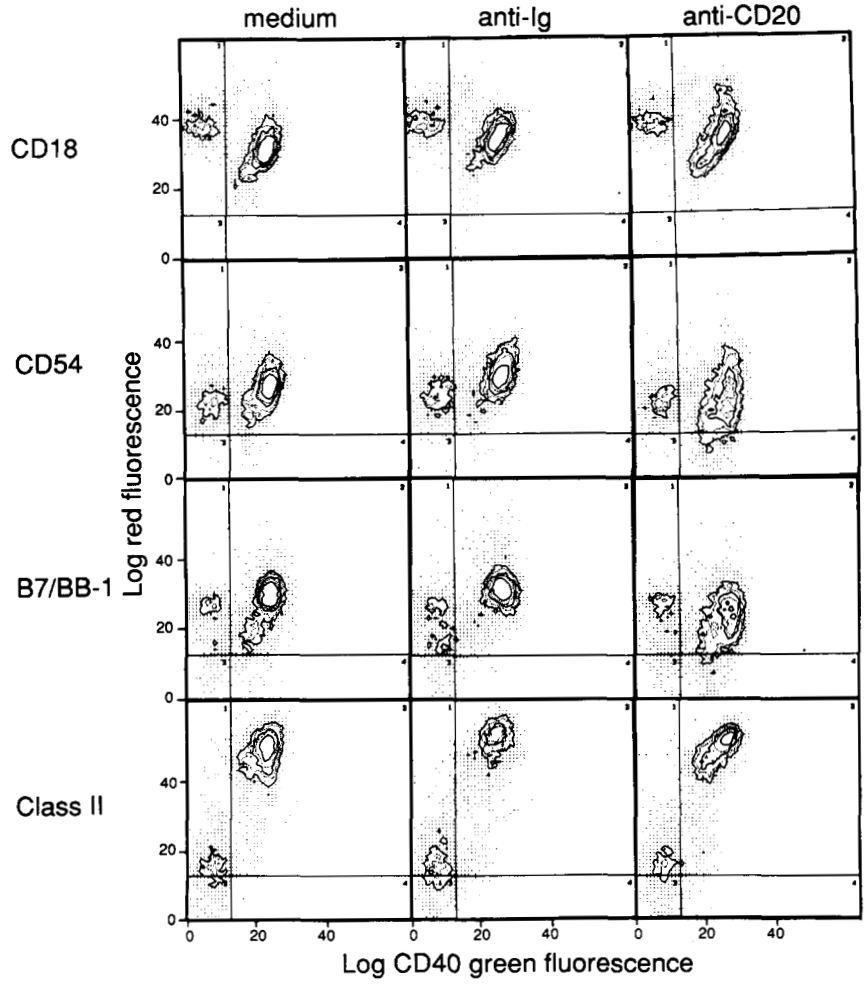

Figure 5. Effect of treatment with anti-IgM or anti-CD20 on the expression of adhesion/recognition molecules on human B cells. Two-color flow cytometric analysis of peripheral blood B cells cultured for 48 h with either medium, anti-IgM (5 μg/ml) or the anti-CD20 mAb, 1F5 (5 μg/ml). Cells were stained for green fluorescence to detect the B-restricted marker CD40 and for red to detect either CD18 (LFA-1 β chain), CD54 (I-CAM-1), B7/BB1, or MHC class II. The cells in windows 2 and 4 are $CD40^+$ B cells, and cells in windows 1 and 3 are CD40-non-B cells. Mean peak expression as a percentage of control was as follows: CD18: 256% (anti-IgM) 247% (anti-CD20); CD54: 163% (anti-IgM), 27% (anti-CD20); B7/BB1: 100% (anti-IgM), 61% (anti-CD20); class II: 164% (anti-IgM), 156% (anti-CD20).

pertussis toxins: the CD20 pathway is clearly inhibited by cholera and pertussis toxins whereas these toxins either have no effect or enhance the anti-IgM induction of *c-myc* mRNA. The differential sensitivity of the CD20 and surface-IgM pathways to cholera toxin is unlikely to be due to effects mediated via the induction of cAMP by this toxin because 1) a strong inducer of cAMP, forskolin, had no effect on the ability of anti-CD20 to signal and 2) a potent inhibitor of cyclic nucleotide-dependent kinases, HA1004, had neither an upregulatory nor a down-regulatory effect on CD20 signal transduction. The A subunits of both cholera and pertussis toxins induce the ADP-ribosylation of the α-subunits of heterotrimeric G proteins and thereby irreversibly modify their activity (47, 48). Thus, an intriguing, but by no means proven, possibility is that IgM and CD20 use different GTP-binding proteins that are differentially modified by these toxins. The inability of either toxin to affect the induction of *c-myc* mRNA by PMA, which acts by direct stimulation of PKC, is consistent with this model. Recently, two independent groups have reported evidence for distinct signal pathways which are distinguished by G proteins that are either sensitive or insensitive to pertussis toxin (49, 50).

The CD20 and IgM signals do have some interesting similarities; both pathways, as assayed by induction of *c-myc* mRNA, are blocked by trifluorperazine, which inhibits both PKC and calmodulin-dependent kinases, and by staurosporine, which is a very potent inhibitor of PKC. Down-regulation of PKC by pretreatment of the cells with PMA also interfered with both signals. The inhibitor, W7, was also effective in blocking either pathway, at doses consistent with an inhibition of PKC. Together these results are most consistent with the involvement of PKC in the signaling pathways induced by either anti-IgM or anti-CD20. However, some caution must be exercised in interpreting these data because the inhibitors used and PMA can have biological effects not involving PKC.

Anti-IgM has previously been shown to translocate cytosolic PKC to the plasma membrane, presumably via the breakdown of phosphatidylinositol bisphosphate to diacylglycerol; at the same time the other metabolite of this breakdown, Ins-1,4,5-P_3, mediates the release of calcium from intracellular stores (3, 35, 51, 52). By contrast, anti-CD20 induces only minor changes in inositol phospholipid metabolism and no detectable increase in intracellular calcium (11, 12). The effect of anti-CD20 on inositol phospholipid metabolism is small but not negligible, and it is possible that it is sufficient to induce calcium/PKC signaling. Many studies of the Ins-P_3-mediated calcium release determine the mean calcium of cell populations. It has been widely observed that the calcium elevation after Ins-1,4,5-P_3 production occurs in the form of an oscillation of calcium concentration the periodicity of which varies between cells and that may be sustained for a considerable length of time (36). The averaging of

the individual cell calcium levels to give mean population data results in an underestimate of the peak calcium levels attained in cells, to the extent that a low level oscillation of calcium with relatively long periodicity may not alter the population calcium to detectable levels.

Alternatively, the minimal changes in Ins-P levels seen with anti-CD20 may be due to an indirect effect of anti-CD20 activation on the basal metabolism of the various isomers. The twofold induction of Ins-1,4,5-P_3 by PMA suggests that activation of PKC alters the baseline metabolism of this pathway. The PKC activation induced by anti-CD20 could be independent of phosphoinositide breakdown. Recently, Rosoff et al. (53) have reported that IL-1 can trigger increases in diacylglycerol and PKC activation via the breakdown of phosphatidylcholine. CD20 may signal cells via an analogous mechanism. It is noteworthy that IL-1 has been reported to be costimulatory with anti-IgM with B cells (54, 55) but it is also constimulatory with anti-CD20 (10) and does not induce *c-myc* in B cells (Fig. 1*B*, *lane 3*). The details of the apparent activation of PKC by anti-CD20 and the importance, if any, of the minimal increase in the metabolism of inositol phospholipids in this activation remain to be resolved.

In several aspects the stimulation of B lymphocytes by the mitogen, LPS, is similar to anti-CD20. The addition of LPS to B cells does not lead to increases in intracellular calcium or active inositol phospholipid breakdown (34), however, there is some evidence that LPS activates PKC (51, 56). LPS has also been shown to activate a pertussis toxin-sensitive G protein in WEHI-231 (B lymphoma) and $P388D_1$ (macrophage) cells (57). Pertussis toxin blocks the ability of LPS to stimulate IL-1 production in $P388D_1$ cells at doses consistent with the modification of a Gi-like G protein (58). Pretreatment of these cells with pertussis toxin also prevents the inhibitory effect of LPS on adenylate cyclase in vitro and the addition of LPS to membrane preparations from either cell type blocks the subsequent labeling, by pertussis toxin, of a 41,000 m.w. protein with [^{32}P]-NAD (57). Inasmuch as only inactive G protein is a substrate for pertussis toxin, these results suggest that the pretreatment with LPS activates a Gi-like protein thus, reducing the available substrate for ADP-ribosylation.

A question remains as to whether IgM and CD20 activate resting B cells by distinct pathways in the same cell or, as we have suggested previously (10), normally sequential and linked signaling systems with CD20 preceding and preparing B cells for signal by Ag. Although small, dense, phenotypically homogeneous and resting B cells were used in these studies, the B cells nonetheless may be heterogeneous in their responsiveness to anti-CD20 or anti-IgM. Therefore, we cannot formally rule out the possibility that different subsets of resting B cells were triggered by anti-IgM and anti-CD20 to express *c-myc*. There is evidence, however, that cross-linking of IgM can directly effect the behavior of CD20 in the same cell: 1) the addition of anti-IgM to B cells causes a transient increase in the level of phosphorylation of CD20 (59) and 2) intact IgM mAb, 2C3, inhibits the induction of *c-myc* mRNA levels by anti-CD20 presumably via interaction of surface-Ig and FcR (M. W. White, G. Shu, D.R. Morris, and E. A. Clark, manuscript in preparation). The anti-Ig mAb, 2C3, is therefore affecting CD20 signaling directly.

B cells can be activated to enter the cell cycle not only by anti-CD20 and anti-IgM, but also by cross-linking another B cell surface molecule, Bgp95 (60). Similarly, T cells can be activated by cross-linking one of several surface receptors (19). Why then do lymphocytes require alternative activation pathways? One explanation is that the way B cells are activated affects which set of cell adhesion/recognition molecules are increased. Anti-IgM increases the expression of CD18, CD54, B7/BB1, and class II MHC, whereas anti-CD20 increases the expression of CD18 and class II, but reduces the expression of B7/BB1 and CD54 (this study) (45, 46). Furthermore, PMA, unlike anti-IgM, is relatively ineffective at increasing or decreasing expression of B7/BB1 on resting B cells (45) but does stimulate increased expression of class II, CD5, CD18, CD40, and CD54 (44, 46). Similarly, anti-CD40 mAb activates resting B cells to express increased levels of CD54 but not to increase CD18 levels.[4] Each of these signals (anti-IgM, anti-CD20, PMA, and anti-CD40) also induces distinctive early events in B cells (46) (see footnote 4), but the full details of how selective sets of cell adhesion/recognition molecules are induced on B cells remains to be elucidated.

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