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Immunology

B-lymphocyte activation mediated by anti-immunoglobulin antibody in the absence of protein kinase C

(surface immunoglobulin crosslinking/8-mercaptoguanosine/phorbol 12-myristate 13-acetate)

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ABSTRACT **B-cell activation induced by crosslinking of surface immunoglobulin is known to stimulate hydrolysis of phosphatidylinositol to diacylglycerol and inositol trisphosphate. We now provide evidence that alternative pathways of activation may also be recruited during such activation. We utilized depletion of protein kinase C activity to determine whether this enzyme is required under all conditions for anti-immunoglobulin-stimulated B-cell activation. Although anti-immunoglobulin does not induce B-cell proliferation in protein kinase C-depleted cells, it stimulates an earlier event in B-cell activation as reflected by its ability to enhance the expression of major histocompatibility complex-encoded class II molecules. Furthermore, the ribonucleoside 8-mercaptoguanosine restores the ability of anti-immunoglobulin to induce B-cell proliferation in protein kinase C-depleted cells. This restoration is also demonstrated by an enhancement of synthesis of a nuclear protein that we find is increased during B-cell mitogenesis. These results indicate that B-cell activation stimulated by anti-immunoglobulin may recruit pathways in addition to the one dependent on protein kinase C.**

Despite extensive investigations into the mechanisms of action of various mitogenic substances that stimulate B lymphocytes to enter G_1 or S phase of the cell cycle, elucidation of their activational pathways remains elusive. It is clear that different mitogens may utilize different pathways to activate B cells. Activation of B cells by crosslinking of surface immunoglobulin (sIg), either by anti-Ig antibodies or by antigens, has been shown to stimulate an endogenous phospholipase C to hydrolyze phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate, which in turn triggers mobilization of intracellular Ca^{2+}, and 1,2-diacylglycerol, which synergizes with Ca^{2+} to activate protein kinase C (PKC) (1–6). Inhibition of inositolphospholipid metabolism and of the increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) by pretreatment with phorbol 12-myristate 13-acetate (PMA) was recently shown to block the mitogenic signal induced by anti-Ig (5, 7), a finding that confirms the observations by Coggeshall and Cambier (2), who reported that these biochemical events are crucial in the cascade of events leading to B-cell proliferation in response to sIg crosslinking. Other mitogens, as for example bacterial lipopolysaccharide, that stimulate comparable levels of B-cell proliferation stimulate neither increases in $[Ca^{2+}]_i$ nor turnover of inositolphospholipid (7). Similarly, activation of B cells by the lymphokine interleukin 4 (IL-4), which stimulates enhanced expression of major histocompatibility complex (MHC)-encoded class II molecules (8, 9), also fails to activate PKC or to induce an increase in $[Ca^{2+}]_i$ (5). These observations that B cells can be stimulated by mitogens or lymphokines via modes of activation that are independent of $[Ca^{2+}]_i$ and PKC suggested to us that anti-Ig antibody may also be capable of stimulating B cells by PKC-independent pathways as well as by a PKC-dependent mechanism. Our results indicate that this in fact is the case, as we found that B cells depleted of PKC express full mitogenic responsiveness to anti-immunoglobulin if cultured in the presence of 8-mercaptoguanosine (8sGuo).

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MATERIALS AND METHODS

Mice. DBA/2 mice were purchased from The Jackson Laboratory and used when 8–12 weeks old.

Reagents. *Escherichia coli* lipopolysaccharide (LPS) 011B4 was purchased from List Biological Laboratories (Campbell, CA). 8sGuo was from Sigma. Affinity-purified goat anti-mouse IgD was prepared as described (10). IL-4 was generously provided by W. E. Paul and J. Ohara (Laboratory of Immunology, National Institutes of Health).

B-Cell Purification and Cell Culture. Splenic B cells were enriched by a modification of the method of Liebson *et al.* (11). Spleen cells were treated at 4°C for 45 min with a mixture of monoclonal antibodies including rat anti-Thy-1.2, anti-L3T4, and anti-Lyt-2, followed by treatment for 45 min at 37°C with mouse monoclonal anti-rat κ light chain (MAR 18.5, ATCC) and newborn rabbit complement (1:5 dilution). These B cells were free of T cells, as shown by the absence of positively stained cells following staining with fluorescein-labeled anti-Thy-1.2 and analysis with a fluorescence-activated cell sorter. The B-cell preparation showed no proliferation (i.e., [^{3}H]thymidine incorporation) in response to the T-cell mitogen concanavalin A (Con A) after 2, 3, 4, or 5 days of *in vitro* culture. Small, resting B cells were subsequently obtained by centrifugation over a discontinuous Percoll density gradient with Percoll (Pharmacia) concentrations (wt/wt) of 50%, 60%, 65%, and 70%. The fraction that layered over 70% Percoll (small, resting B cells) was used in all experiments. Cells were cultured in Mishell–Dutton medium (12) supplemented with 10% endotoxin-free fetal bovine serum (Hyclone, Logan, UT) and 50 μM 2-mercaptoethanol, in flat-bottomed microwell plates (Costar, Cambridge, MA) at densities of $0.5–2 \times 10^5$ in 0.2 ml of medium per well. Plates were incubated in a humidified 5% CO_2 incubator at 37°C for the indicated times with the various mitogenic combinations. [*methyl*-^{3}H]Thymidine was added (1.0 μCi per well; 1 μCi = 37 kBq) 18 hr before the cells were harvested; the time of

Abbreviations: IL-4, interleukin 4; $[Ca^{2+}]_i$, intracellular concentration of free Ca^{2+}; sIa, surface immune response-associated antigen; sIg, surface immunoglobulin; 8sGuo, 8-mercaptoguanosine; LPS, lipopolysaccharide; MHC, major histocompatibility complex; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate.

harvest was recorded as the time of assay. Triplicate cultures were assayed, and results are expressed as the arithmetic mean ± SEM of counts per min (cpm) per culture.

Radiolabeling of Cellular Proteins. Radiolabeling of cellular proteins was done as described (13). Cells were washed in phosphate/saline buffer, resuspended (5×10^6 per ml) in methionine-free RPMI 1640 medium supplemented with 5% dialyzed fetal bovine serum (GIBCO) and [^{35}S]methionine (80–100 μCi/ml), and incubated for 4 hr at 37°C.

Isolation of Nuclei and Analysis of Nuclear Proteins. Nuclei were isolated as described (14). Cells (10^7) were washed in phosphate/saline buffer (pH 7) and suspended in 5 ml of hypotonic nuclear buffer containing 10 mM Tris·HCl (pH 8.0), 3 mM $CaCl_2$, 0.25 M sucrose, and 0.1 mM phenylmethylsulfonyl fluoride. The cells were disrupted by gentle homogenization. The lysates were centrifuged at $1000 \times g$ for 5 min and the nuclear pellets were washed twice with nuclear buffer containing 0.5% Nonidet P-40 (NP-40). Isolation of pure nuclei was confirmed by phase-contrast microscopy. Whole nuclei were suspended in isoelectric focusing lysis solution [9.5 M urea/2% NP-40/5% 2-mercaptoethanol/2% Ampholines (pH 3.5–10; LKB)]. After vigorous Vortex mixing, the insoluble material was removed by centrifugation and the sample was subjected to two-dimensional (isoelectric focusing, $NaDodSO_4$) PAGE, staining, and radiofluorographics as described (15). Standardization of control and experimental conditions was by analysis of equal amounts of trichloroacetic acid-precipitable cpm by two-dimensional PAGE. Immunoblot analysis of PKC was done as described (16). B cells (5×10^6) were lysed in 20 mM Tris·HCl, pH 7.5/1.0 mM dithiothreitol/0.5 mM EDTA/0.5 mM EGTA/10% glycerol/0.5% NP-40. Proteins were fractionated by $NaDodSO_4$/10% PAGE and electrophoretically transferred to a nitrocellulose membrane. Immunoblotting was carried out by using a 1:1000 dilution of immune serum against rat brain PKC. This antibody reacts with all known species of protein kinase C (16). Detection of immunoreactive bands was done by incubation with ^{125}I-labeled protein A followed by autoradiography of the nitrocellulose membrane for 24 hr. The membrane was then stained with amido black in 25% isopropyl alcohol containing 10% acetic acid.

RESULTS

Anti-Ig Antibody Stimulation of Expression of MHC Class II Molecules (Ia) in PKC-Depleted B Cells. Anti-Ig stimulation of B-cell surface expression of MHC class II molecules (sIa) reflects a relatively early event in B-cell activation (17, 18). We wished to determine whether this early activational step could be stimulated in PKC-depleted B cells, in view of the recent report (5) that such B cells cannot proliferate in response to anti-Ig. Percoll-separated small B cells were cultured with PMA (100 ng/ml) or with medium alone for 24 and 48 hr, after which anti-IgD was added for an additional 18 hr (Table 1). To verify that PKC was depleted by incubation with PMA, we employed immunoblot analysis (Fig. 1) and found that, as previously reported (19, 20), PMA treatment for 18 hr caused a complete depletion of PKC (compare lanes 1 and 2 in Fig. 1*B*). Measurement of sIa expression showed that PMA alone caused an increase in the expression of sIa, as previously demonstrated (21). The addition of anti-IgD to cells that had been precultured with PMA for 24 or 48 hr stimulated an even greater increase in the expression of sIa, equivalent to that stimulated by mitogens in cells not pretreated with PMA (Table 1). Similarly, LPS and Ca^{2+} ionophore A23187, which do not activate B cells via the stimulation of phosphatidylinositol hydrolysis, also stimulated increases in expression of sIa in the PKC-depleted B cells. These PKC-depleted cells, however, could not be stimulated to proliferate in response to anti-IgD; pretreatment of the cells with PMA for 24 hr caused a 95% inhibition in thymidine incorporation induced by anti-IgD (Table 1). PKC depletion did not significantly inhibit the induction of thymidine incorporation induced by the PKC-independent mitogen LPS in the majority of experiments. In a small number of experiments (3/9), PKC depletion caused a 20–30% decrease in the LPS-stimulated response if responsiveness was measured as Δcpm (i.e., experimental cpm − control cpm). However, if the magnitude of LPS-induced responses was measured as E/C [i.e., (experimental cpm)/(control cpm)], no difference was noted in the LPS-stimulated responses of PKC-depleted and normal B-cell populations. These results demonstrate that although PKC is required for anti-Ig-stimulated mitogenesis, it is not required for anti-Ig-enhanced expression of sIa.

Addition of 8sGuo Can Restore Responsiveness to Anti-IgD in PKC-Depleted B Cells. 8sGuo has been reported to stimulate proliferation in partially activated B cells but not in resting B cells (22). We have found that anti-Ig but not other B-cell activators (IL-4 or anti-Lyb-2.1) can activate resting B cells to a stage at which 8sGuo can exert its mitogenic activity (unpublished observation). We therefore wished to determine whether anti-IgD could also stimulate PKC-depleted B cells to an activational stage where such synergy with 8sGuo could be observed. Cells were cultured with or without PMA for 18 hr and washed; then anti-IgD, IL-4, and 8sGuo (singly or in combination) were added and the cells were cultured for an additional 48 hr. PKC-depleted B cells did not proliferate

Table 1. Effect of PMA pretreatment on mitogen stimulation of sIa expression and DNA synthesis in B cells

	sIa expression, median fluorescence intensity					
	One-day preincubation		Two-day preincubation		[^{3}H]Thymidine incorporation, cpm	
Mitogen	Medium	PMA	Medium	PMA	Medium	PMA
None	82	192	77	138	1,045	353
Anti-IgD	423	445	530	632	29,516	949
LPS	436	705	440	796	70,523	45,031
A23187	276	269	405	328		

Small, resting B cells were cultured at 2×10^6 per ml with medium or PMA (100 ng/ml) for 1 or 2 days prior to addition of goat anti-mouse IgD (10 μg/ml), LPS (50 μg/ml), or A23187 (200 ng/ml). Eighteen hours later, an aliquot was stained with fluorescein-conjugated MKD6 (anti-Ia^d) and evaluated by flow cytometry for fluorescence intensity. The remaining cells were incubated with [^{3}H]thymidine for 18 hr and then harvested on glass-fiber sheets (PhD cell harvester, Cambridge Technology) for evaluation of thymidine incorporation by liquid scintillation spectrometry. The median fluorescence intensity represents that fluorescence channel above and below which lie 50% of positively stained cells. In this 1 of 4 representative experiments, 98% of cells were sIa$^+$.

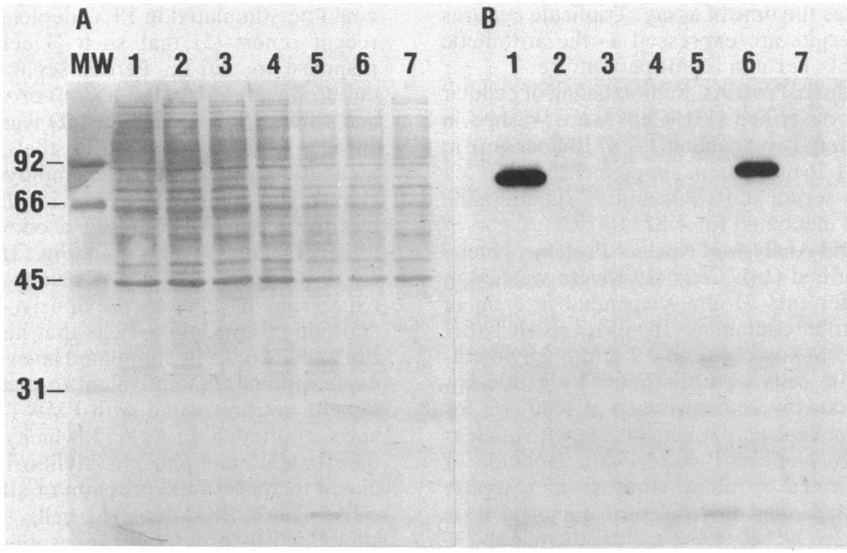

FIG. 1. Absence of PKC in PMA-treated cells stimulated by anti-IgD and 8sGuo. Cells were treated with PMA for 18 hr before addition of anti-IgD and 8sGuo. Cell lysates were subjected to $NaDodSO_4$/PAGE and the separated proteins were transferred to nitrocellulose for immunoblot detection with anti-PKC and ^{125}I-labeled protein A. (*A*) Amido black staining. (*B*) Autoradiography. Lane 1: control cells incubated with medium for 24 hr. Lane 2: cells treated with PMA (100 ng/ml) for 24 hr. Lanes 3–5: PMA-treated cells incubated with anti-Ig plus 8sGuo for 5, 24, and 48 hr, respectively. Lane 6: control cells incubated with medium for 48 hr. Lane 7: cells incubated with PMA for 48 hr. Lane MW (in *A*): molecular weight standards ($M_r \times 10^{-3}$ at left).

in response to IL-4, 8sGuo, or anti-IgD or to anti-IgD in combination with IL-4 but did when stimulated by a combination of anti-IgD and 8sGuo (Table 2). The response to anti-IgD plus 8sGuo was similar in the untreated cells and in the PKC-depleted B cells (Table 2). Identical results were obtained when PMA was incubated continuously with the B cells (data not shown).

To examine whether restoration of the anti-Ig-stimulated response by 8sGuo is associated with induction of PKC, we examined PKC in PMA-pretreated cells stimulated by the mitogenic combination of anti-IgD plus 8sGuo. Cells were pretreated with PMA for 18 hr and then incubated with anti-Ig and 8sGuo for 5, 24, and 48 hr and lysed. Proteins were separated by $NaDodSO_4$/PAGE, electrophoretically transferred to nitrocellulose, and immunoblotted with antibody against PKC (16). Stimulation of PKC-depleted cells (i.e., cells pretreated with PMA for 18 hr) with anti-IgD and 8sGuo did not restore the immunoreactive PKC in the cellular protein pool (Fig. 1, lanes 3–5). Parallel cultures set up for study of [^{3}H]thymidine incorporation indicated that the enhanced DNA synthesis could be detected 24–48 hr after addition of anti-IgD and 8sGuo (data not shown). These results indicate that the mitogenesis induced in the PKC-depleted cells by anti-IgD and 8sGuo does not involve restoration of the intracellular pool of PKC.

Since anti-Ig can stimulate turnover of phosphatidylinositol bisphosphate and elevations in $[Ca^{2+}]_i$ in cells pretreated with PMA for 24 hr (W. E. Paul, personal communication; unpublished observations), it was possible that its ability to stimulate increases in $[Ca^{2+}]_i$ accounted for its ability to synergize with 8sGuo. To test this, we cultured control or PKC-depleted B cells with Ca^{2+} ionophore A23187 in the presence of 8sGuo. While A23187 at 200 ng/ml stimulates increases in $[Ca^{2+}]_i$ that are 6-fold above background levels (unpublished observations), this by itself was not sufficient to enable the cells to proliferate in response to 8sGuo (Table 3). This experiment was repeated twice more with A23187 concentrations varying from 25 to 300 ng/ml. In neither case was synergy with 8sGuo noted.

We have recently observed that stimulation of B cells with anti-Ig antibody, as well as with other mitogens, is characterized by a prominent increase in the synthesis of a M_r 40,000 nuclear protein with a pI of 5 (p40/pI5). To determine whether the synthesis of this protein could also be stimulated by the combination of anti-IgD and 8sGuo in PKC-depleted

Table 2. Stimulation of B-cell proliferation by the combination of anti-IgD and 8sGuo in PKC-depleted B cells

Mitogen(s)	[^{3}H]Thymidine incorporation, cpm	
	Medium	PMA
None	846 ± 63	527 ± 40
IL-4	1,172 ± 360	860 ± 110
8sGuo	4,250 ± 322	755 ± 35
Anti-IgD	9,386 ± 622	688 ± 66
Anti-IgD + IL-4	48,072 ± 3,341	1,900 ± 320
Anti-IgD + 8sGuo	33,220 ± 1,980	39,835 ± 2,195

Small, resting B cells were cultured at 2×10^6 per ml for 18 hr in medium only or with PMA (100 ng/ml), washed three times in complete medium, and incubated at 5×10^5 per ml in microwells with anti-IgD (100 μg/ml), 8sGuo (0.5 mM), or IL-4 (10 units/ml) or with mitogen combinations as indicated. Eighteen hours later, [^{3}H]thymidine was added; cells were harvested after 18 hr for measurement of thymidine incorporation as described for Table 1.

Table 3. Synergistic stimulatory activity of 8sGuo and anti-IgD in PKC-depleted cells does not result solely from increases in $[Ca^{2+}]_i$ stimulated by anti-IgD

Mitogen(s)	[^{3}H]Thymidine incorporation, cpm	
	Medium	PMA
Medium	700	666
Anti-IgD	7,076	640
8sGuo	1,799	921
A23187	718	413
Anti-IgD + 8sGuo	31,544	31,797
A23187 + 8sGuo	1,615	1,168

B cells were cultured at 2×10^6 per ml for 18 hr in medium only or with PMA (100 ng/ml), washed three times in complete medium, and incubated at 5×10^5 per ml in microwells with 8sGuo (1.0 mM), Ca^{2+} ionophore A23187 (200 ng/ml), or anti-IgD (100 μg/ml) or with mitogen combinations as indicated. Thymidine incorporation was determined as for Tables 1 and 2.

B cells, resting B cells were cultured with or without PMA for 3 hr; anti-IgD, 8sGuo, or both then were added for 16 hr and then the cells were incubated with [^{35}S]methionine (100 μCi/ml) for 4 hr. Nuclei were isolated and the nuclear proteins were analyzed by two-dimensional PAGE (Fig. 2). Radioactivity in the p40/pI5 nuclear protein, quantitated directly in the gels, was 87 cpm in control cells, 539 cpm in anti-IgD-activated cells, 200 cpm in PMA-treated cells activated by anti-IgD, 660 cpm in cells activated with anti-IgD and 8sGuo, and 600 cpm in PMA-treated cells activated with anti-IgD and 8sGuo. Thus, induction of this protein by anti-IgD in PKC-depleted cells was inhibited by 63%, but addition of 8sGuo overcame this inhibitory effect. These results provide further evidence that addition of 8sGuo can restore the biochemical events associated with mitogenesis in B cells depleted of PKC.

DISCUSSION

PKC plays an important role in the activation of many different cell types by a wide range of agonists (23). Stimuli that exert their effect via activation of PKC can also stimulate

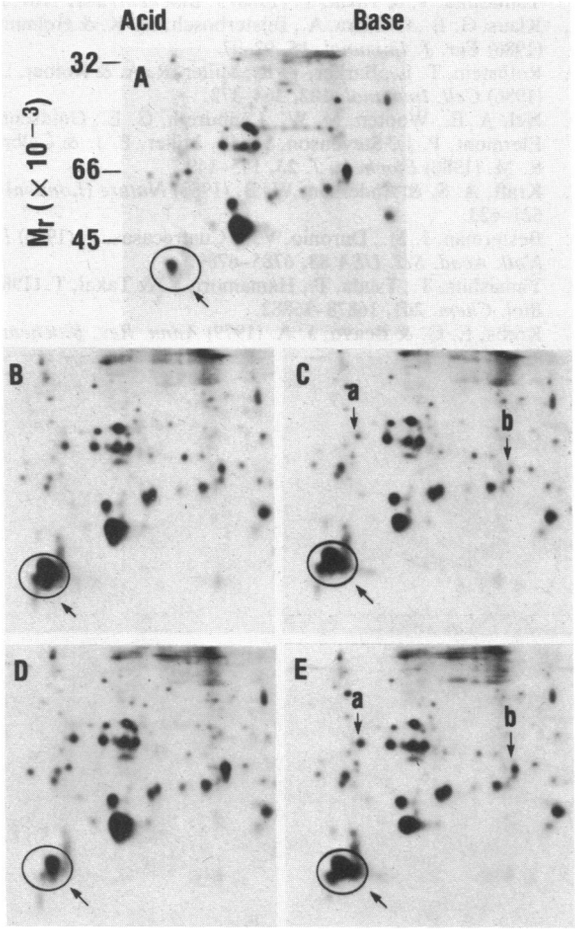

FIG. 2. Inhibition of induction of p40/pI5 nuclear protein by anti-IgD in PKC-depleted B cells and restoration of induction by 8sGuo. Cells were pretreated in the presence or absence of PMA (100 ng/ml for 3 hr), further stimulated by addition of anti-IgD or anti-IgD plus 8sGuo for 16 hr, and then labeled with [^{35}S]methionine for 4 hr. Nuclei were isolated and the nuclear proteins were analyzed by two-dimensional PAGE. Equal cpm were applied to the gels. (*A*) Nontreated, resting cells. (*B*) Anti-IgD-activated cells. (*C*) Anti-IgD/8sGuo-activated cells. (*D*) PMA-pretreated, anti-IgD-activated cells. (*E*) PMA-pretreated, anti-IgD/8sGuo-activated cells. Spot corresponding to p40/pI5 nuclear protein is circled on each fluorograph. Downward arrows (a and b) indicate proteins induced by anti-IgD/8sGuo in the PMA-treated cells.

similar cellular responses even in the absence of PKC (24–26). Kaibuchi *et al.* (27) demonstrated that platelet-derived growth factor, which had been shown to induce activation of PKC and stimulate an increase in mRNA levels of the protooncogene c-*myc*, can also stimulate an increase in expression of c-*myc* RNA in fibroblasts depleted of PKC. While there are ample data demonstrating that anti-Ig-mediated crosslinking of B-cell sIg initiates a PKC-dependent mode of activation, there is only suggestive evidence to support the possibility that a PKC-independent mechanism could also account for this stimulation. Maximal B-cell proliferative responses are stimulated after a 48-hr culture period with PMA and A23187 (28, 29), a combination that induces intracellular events comparable to those induced by anti-Ig, despite the fact that by 12 hr, PKC is undetectable in these cells (19, 20). This suggests that those B-cell stimuli which mimic anti-Ig-stimulated B-cell activation and which initiate a PKC-dependent pathway may not require the continued presence of PKC to maintain enhanced levels of proliferation. An additional suggestion that anti-Ig may either stimulate PKC-independent activation or maintain B-cell activation in the absence of PKC is the observation that B cells must be cultured with anti-Ig for 36–48 hr to induce proliferation. Since anti-Ig has been shown to induce rapid translocation of PKC to the membrane, with subsequent degradation, little if any PKC remains by 24 hr (4, 30). The requirement for anti-Ig beyond this point does not simply reflect a requirement for maintenance of elevated $[Ca^{2+}]_i$, since the ionophore A23187 cannot replace its function (unpublished observations). For these reasons and in view of the fact that LPS has been reported to stimulate a PKC-independent mode of B-cell activation (7), we examined more carefully whether anti-Ig could also stimulate a PKC-independent pathway leading to B-cell activation. The finding that pretreatment of B cells with PMA, which results in PKC depletion, does not prevent anti-Ig-mediated increases in B-cell sIa expression or the ability of anti-Ig to synergize with 8sGuo to stimulate B-cell proliferation suggests that the presence of PKC may not be required for all anti-Ig-mediated signaling. However, this finding does not exclude the possibility that very low levels of PKC, undetectable by our assay system, may be involved or that PKC activation may be required as an initial step in this anti-Ig-stimulated pathway, since treatment with PMA results in PKC activation prior to its degradation (31). Indeed, anti-Ig by itself may be unable, in the absence of PKC, to activate the cells to a stage where they are responsive to 8sGuo, and it may be that only the combination of anti-Ig and 8sGuo can function like LPS as a mitogenic stimulus for B cells in the absence of PKC. Although this does not clarify how anti-Ig-mediated stimulation differs mechanistically from stimulation mediated by anti-Ig plus 8sGuo, there are a number of intriguing possibilities. (*i*) Anti-Ig may activate a phosphatidylcholine-specific phospholipase C (32); in the absence of PKC, phosphocholine or diacylglycerol produced from phosphatidylcholine may serve as a second messenger to promote B-cell activation in the presence of 8sGuo. (*ii*) Diacylglycerol, which is formed by the hydrolysis of either inositolphospholipids or phosphatidylcholine, is metabolized to arachidonic acid, from which other biologically active derivatives, including prostaglandin E, are produced. It has been reported (33) that E-type prostaglandins, when synthesized, leave the cell and stimulate cAMP synthesis through interaction with their own receptor. This increase in cellular levels of cAMP has been shown to constitute a growth-promoting signal for other cell types (34), and it may be this pathway that acts synergistically with 8sGuo to stimulate B-cell DNA synthesis. (*iii*) The anti-Ig-induced increases in $[Ca^{2+}]_i$ may stimulate Ca^{2+}/calmodulin-dependent protein kinase B, which together with the above-mentioned path-

ways may contribute to synergy with 8sGuo. Although these possibilities are only speculative, they emphasize the point that sIg crosslinking may contribute to B-cell activation through pathways other than those requiring PKC.

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