Surface immunoglobulin-mediated B-cell activation in the absence of detectable elevations in intracellular ionized calcium: A model for T-cell-independent B-cell activation

(Signal transduction/phosphatidylinositol bisphosphate hydrolysis)

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ABSTRACT We recently showed that anti-immunoglobulin conjugated to high molecular weight dextran is 1000-fold more mitogenic for B cells than unconjugated anti-immunoglobulin. This system serves as a model for T-cell-independent type 2 antigens such as haptenated Ficol, dextran, and bacterial polysaccharides, which can also stimulate B-cell proliferation and antibody production at low concentrations. We show here that conjugated anti-immunoglobulin, at concentrations that stimulate significant increases in expression of major histocompatibility complex class II molecules and incorporation of thymidine into DNA, does not induce detectable modulation of surface immunoglobulin. These results indicate that the facilitated T-cell-independent B-cell activation by polysaccharide antigens may result from inability to modulate surface immunoglobulin, possibly resulting in persistent and/or repetitive signaling. Early large increases in \([Ca^{2+}]\) and breakdown of inositol phospholipids presently thought to be involved in transduction of the mitogenic signal are not detectable at low concentrations of conjugated anti-immunoglobulin, raising the possibility that these biochemical events may not in fact be central to this signaling pathway.

Crosslinking of B-cell-surface immunoglobulin by antigen or anti-Ig antibody has been demonstrated to induce phospholipase C-dependent phosphatidylinositol bisphosphate (PIP2) hydrolysis (1–6). The second messengers thereby generated, diacylglycerol (DAG) and inositol trisphosphate (IP3), lead to activation of protein kinase C (PKC) and mobilization of intracellular ionized calcium ([Ca^{2+}]) (reviewed in refs. 7, 8), events that facilitate entry of B cells into G1 and S phases (9–14). Stimulation of B cells by other agonists does not appear to stimulate an inositol phospholipid-dependent mode of activation. Thus, stimulation of murine B cells with lipopolysaccharide or 8-mercaptoguanosine leads to DNA synthesis in the absence of detectable increases in \([Ca^{2+}]\); (ref. 15 and our unpublished observations). Likewise, stimulation of human B cells with antibodies to the CD20 cluster (16) or to Bgp95 (17) either alone or in combination does not stimulate increases in \([Ca^{2+}]\), indicating that many alternative routes of activation can be recruited by B cells after stimulation by agonists that do not bind to surface immunoglobulin (slg).

Recently we showed that when anti-Ig antibody is conjugated to high molecular weight dextran (anti-Ig-dex) it stimulates B-cell proliferation at concentrations 1000-fold lower than that stimulated by unconjugated anti-Ig antibody (18). Furthermore, at these low concentrations anti-Ig-dex induces greater B-cell proliferation than that induced by unconjugated anti-Ig but induces no modulation of slg and very low levels of binding to slg. This suggests that slg-modulated B-cell activation could be stimulated despite low-level receptor occupancy and led us to investigate whether these conjugates stimulate B-cell activation via stimulation of PIP2 hydrolysis similar to that induced by high concentrations of unconjugated anti-Ig antibody. The data show that anti-Ig-dex stimulates greater accumulation of inositol phosphates and greater increases in \([Ca^{2+}]\) as compared to comparable concentrations of anti-Ig. However, at lower concentrations that remain fully mitogenic we can detect no such increases. These data indicate that early large increases in \([Ca^{2+}]\) may not be essential for slg-mediated B-cell activation.

MATERIALS AND METHODS

Mice. Female DBA/2J mice (from The Jackson Laboratories), 8 weeks old, were used within 4 weeks of arrival. Antibody and Other Reagents. Anti-T-cell antibodies for B-cell purification were anti-Thy1.2 (clone 30-H12), anti-L3T4 (clone GK1.5), anti-Lyt-2 (clone 53-6.7), and rat anti-mouse immunoglobulin \(\kappa\) chain (clone MAR18.5). Other antibodies were anti-Fc-\(\gamma\) receptor (clone 24G2), anti-IgD of \(a\) allototype (clone H5/8/1 and clone FF4), fluoresceinated anti-\(\lambda\) (clone MKD6), and fluoresceinated anti-IgD of the \(\alpha\) allototype (clone AMS15), and IgD (clone TEPC1017). All antibodies except 53-6.7 and MAR18.5, which were used as crude ascitic fluid and tissue culture fluid, respectively, were purified by precipitation with \((NH_4)_2SO_4\), DE-52 ion-exchange chromatography, and filtration with Sephadex G-200 gel as described (18). Indo-1AM was from Molecular Probes, and Dowex 1 formate was from Bio-Rad. \(^{3}H\)-Thymidine (specific activity, 20 Ci/mmole; 1 Ci = 37 GBq) and myo-\(^{3}H\)inositol were from New England Nuclear.

B-Cell Purification. Resting B cells were purified as described (18): spleen cell suspensions were depleted of T cells by a cocktail of anti-T cells (30-H12, GK1.5, 53-6.7) followed by treatment with rabbit complement in the presence of MAR18.5. Resting B cells were separated on a discontinuous Percoll gradient (Pharmacia). Cells banding at the 65–70% interface were then isolated and used as resting cells.

Antibody-Dextran Conjugates. Antibodies were coupled to dextran (\(M_f\), 2 × 10^6) by the heteroligation technique recently described (18).

Abbreviations: PKC, protein kinase C; \([Ca^{2+}]\), intracellular ionized calcium; anti-\(\delta\), antibody to the heavy chain of IgD; dex, dextran; anti-\(\delta\)-dex, anti-\(\delta\) coupled to dextran; PIP2, phosphatidylinositol bisphosphate; DAG, diacylglycerol; IP3, inositol trisphosphate; slg, surface immunoglobulin; MHC, major histocompatibility complex.

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FIG. 1. Calcium fluxes in B cells stimulated by anti-δ-dex and anti-δ. Resting B cells were analyzed for 1 min to establish an appropriate baseline after which ligand was added at the indicated concentration of anti-δ-dex (A) or anti-δ (B) and analysis was continued for 6 min. The ratio of violet:blue was converted to Ca concentration (nM). Results are expressed as the mean Ca levels of the population vs. time. Control samples were run with medium, dex, or 2G2-dex with similar results. Cells were stimulated by various concentrations of anti-δ-dex conjugates and analyzed for the indo-1 violet:blue fluorescence ratio (directly proportional to [Ca<sup>2+</sup>]), which was digitally calculated in real time for each individual cell and plotted on the y axis against time on the x axis (C). Data were accumulated from ~500 cells per sec and the ratio within each cell was plotted. Samples are analyzed for 6 min 40 sec and each channel represents data for 4 sec. Results are displayed on a 100 × 100 pixel grid on which the number of cells per pixel is represented by a 16-color progression according to an exponential function in which 0 to 1 cell is assigned a color of black, 2–10, shades of blue, 11–80, shades of green, and 81–600, shades of yellow to red. The gap in analysis occurred when ligand was added to the suspension.
Analysis of \( [\text{Ca}^{2+}]_i \). B lymphocytes were labeled at \( 5 \times 10^7 \) cells per ml with 1.5 \( \mu \text{M} \) indo-1 for 30 min, washed, and analyzed for \( [\text{Ca}^{2+}]_i \) as described in detail (19). Five hundred cells per second were analyzed for \( [\text{Ca}^{2+}]_i \); responses were independent of the cell concentration used. The violet-blue ratio obtained from the cytofluorograph was converted to calcium concentration (nM) and digitally calculated in real time for each individual cell. In some experiments the cumulative calcium influx was determined by finding the area under the curve of "mean \( [\text{Ca}^{2+}]_i \) vs. time" after stimulation and is expressed as \( \mu \text{M}-\text{sec} \) of calcium. Net cytoplasmic calcium elevation in the 6 min after stimulation with medium is subtracted from the net cytoplasmic calcium elevation induced by ligands.

Proliferative Responses. B-cell proliferation was determined by culturing \( 10^5 \) cells per well in 96-well flat-bottom microtiter plates for 48 hr in Mishell–Dutton medium. One micromole of \( ^{3} \text{H} \)-thymidine was added for the last 18 hr of culture and proliferative responses were determined by harvesting onto glass fiber strips (PFD, Cambridge, MA). Specific incorporation was analyzed by liquid scintillation spectroscopy and results are expressed as mean CPM of thymidine incorporation in triplicate wells; standard deviation from the mean was always \(<10\% \) and has been omitted from the figures for the sake of clarity (18).

**DISCUSSION**

Results and Discussion

These studies were undertaken to determine whether low concentrations of anti-IgD-dex (anti-\( \delta \)), which stimulated high levels of B-cell proliferation (18), also stimulated comparable increases in early events of B-cell activation such as PIP2 breakdown and increases in \( [\text{Ca}^{2+}]_i \) (1–5) induced by high concentrations of unconjugated anti-Ig. Although 1 and 0.1 \( \mu \text{g} \) of anti-\( \delta \)-dex per ml stimulated increases in \( [\text{Ca}^{2+}]_i \) in \( >95\% \) of B cells from a resting level of 130 nM to 864 nM and 690 nM, respectively, 100-fold higher concentrations of unconjugated anti-\( \delta \), (100 and 10 \( \mu \text{g} \)) stimulated increases only to 439 nM and 291 nM, respectively (Fig. 1), and the increases in \( [\text{Ca}^{2+}]_i \) were more sustained than that by unconjugated anti-\( \delta \) (Fig. 1). Low concentrations of anti-\( \delta \)-dex, which were highly mitogenic, stimulated barely detectable increases in levels of \( [\text{Ca}^{2+}]_i \); measured early (6 min) or later (3.5 and 4.5 hr) (Table 1). In the experiment shown in Fig. 2, \( 10^{-4} \) \( \mu \text{g} \) of anti-\( \delta \)-dex per ml stimulated thymidine incorporation, which was \( \approx 35\% \) as great as the maximal response, but resulted in an increase in \( [\text{Ca}^{2+}]_i \) that was \(<4\% \) of that observed when cells were maximally stimulated. At this low concentration \( (10^{-3} \mu \text{g} / \text{ml}) \) of anti-\( \delta \)-dex, \( 25\% \) of the B cells were in the G2, S, and M stages of the cell cycle (as determined by propidium iodide staining) compared to \( 43\% \) of B cells that were stimulated with \( 10^{-1} \mu \text{g} / \text{ml} \) (data not shown). This suggests that the high level of thymidine incorporation that we observe does not simply reflect a small number of B cells that synthesize large amounts of DNA but rather reflects a large fraction of the B cells that are induced to enter the cell cycle. Concentrations of anti-\( \delta \)-dex that stimulated slight or no detectable early increases in B-cell \( [\text{Ca}^{2+}]_i \) were much more mitogenic than concentrations of unconjugated anti-\( \delta \) that induced substantial \( [\text{Ca}^{2+}]_i \) increases (Fig. 2). The data in Fig. 2 were derived by integrating the area under the curve of "mean calcium vs. time" (from an experiment similar to that described in Fig. 1). Since this computation is a summation of the mean of the calcium transients from analysis of each individual cell analyzed over the duration of the experiment (6 min), it provides a very accurate and sensitive way of detecting small but potentially significant changes in a population of B cells. The assay system that we employ analyzes events in single

<table>
<thead>
<tr>
<th>Stimulus, ( \mu \text{g} / \text{ml} )</th>
<th>( [\text{Ca}^{2+}]_i ), nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>130.8</td>
</tr>
<tr>
<td>anti-( \delta )-dex</td>
<td></td>
</tr>
<tr>
<td>( 10^{-2} )</td>
<td>228.5</td>
</tr>
<tr>
<td>( 10^{-3} )</td>
<td>161.7</td>
</tr>
<tr>
<td>( 10^{-4} )</td>
<td>130.1</td>
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</tbody>
</table>

Resting B lymphocytes were cultured at \( 37^\circ \)C with medium only or with anti-\( \delta \)-dex at \( 10^{-2} \), \( 10^{-3} \), or \( 10^{-4} \) \( \mu \text{g} / \text{ml} \) for 3.5 or 4.5 hr. Thirty minutes before the end of the culture period indo-1 was added to the cells and they were analyzed for the indo-1 ratio of violet/blue fluorescence. Histograms were generated to show the distribution of fluorescence within the population and were then further analyzed to determine the mean \( [\text{Ca}^{2+}]_i \), of the population. Results are expressed as mean \( [\text{Ca}^{2+}]_i \), in nM.

*Stimulation time.

Table 1. Analysis of the mean \( [\text{Ca}^{2+}]_i \), in individual B cells stimulated for 3.5 and 4.5 hr with anti-\( \delta \)-dex antibodies

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**FIG. 2.** Comparison of anti-Ig-mediated stimulation of increases in \( [\text{Ca}^{2+}]_i \) and thymidine incorporation in B lymphocytes. To compare directly changes in \( [\text{Ca}^{2+}]_i \) and anti-Ig-mediated proliferative responses, resting B cells were analyzed for cumulative flux by determining the area under the curve, plotted for mean \( [\text{Ca}^{2+}]_i \) vs. time after stimulation, and expressed as \( \mu \text{M}-\text{sec} \) of calcium. Net cytoplasmic calcium elevation that occurred in the 6 min after stimulation with medium is subtracted from that induced by various anti-\( \delta \) and anti-\( \delta \)-dex concentrations. Cells from the same preparation were cultured in triplicate in 96-well microculture plates at \( 10^5 \) cells per well and indicated concentrations of anti-\( \delta \)-dex or anti-\( \delta \) were added, specific incorporation was determined, and results were plotted as the mean cpm of thymidine incorporation of triplicate cultures.
cells and is capable of detecting responses in <1% of a population of cells (19). The data shown were derived from individually determined responses of ~500 cells analyzed per second. Although we do not measure calcium transients in individual cells over real time as was done by Wilson et al. (20), we do analyze the response of ~180,000 cells over the course of a 6-min experiment. The dot plot (Fig. 1C) displays the response of individual cells and demonstrates that the reduction of data to the mean is justified. When 24G2 was added to the cultures containing anti-δ or anti-δ-dex to minimize Fc-γ receptor-mediated inhibition (21), there was no qualitative difference in the results. These data indicate that mitogenicity mediated by crosslinking of slg with anti-δ-dex appears to be independent of the levels of stimulated increases in [Ca2+]i.

If the relevant stimulus for an excitatory event in B cells were the establishment of a domain (22) of crosslinked B-cell slg, multivalent ligands, such as anti-δ-dex, would be especially effective in establishing such domains. Thus, a single interaction between a molecule of anti-δ-dex and a B cell might be more likely to trigger excitation than an interaction between the same cell and unconjugated, divalent anti-δ. At the very low concentrations of anti-δ-dex observed as still mitogenic, ligand–receptor interactions might also occur at low levels. Such infrequent and low-level B-cell–ligand interactions, though providing effective signal transduction, as reflected by increased expression of MHC class II molecules, do not modulate much IgD from the B-cell surface at all times tested, including 2, 6, 11 (data not shown), and 24 hr (Table 2) after addition of antibody. Absence of slg modulation increases the likelihood of subsequent interactions of anti-δ-dex with slgD, which would allow repetitive B-cell signaling to occur over longer periods. Lack of a direct relationship between modulation of slg, PIP2 hydrolysis (Table 2), and B-cell activation is supported by the observation that concentrations of anti-δ-dex too low to induce detectable increases in mean B-cell [Ca2+]i, or in breakdown of PIP2 (as measured over 40 min) are also too low to modulate B cell slgD yet induce considerable increases in B-cell expression of class II MHC and B-cell proliferation (Table 2 and Fig. 2). This is not the case when B cells are stimulated with unconjugated bivalent anti-δ, which would theoretically have to be present at considerably greater concentration than anti-δ-dex to crosslink slgD sufficiently to activate B cells. These high concentrations required for B-cell activation are also very effective at modulating B-cell slg (Table 2) and thus may limit their own capacity to activate B cells repetitively.

To examine this hypothesis more carefully we precultured B cells for 2 hr with a nonstimulatory anti-δ antibody (FF1) (23) demonstrated to modulate surface IgD and then added to the cultures anti-δ-dex antibody over a broad range of mitogenic concentrations. We reasoned that the mitogenicity of the lower concentrations of anti-δ-dex antibody was dependent on their relative inability to modulate slgD and hence would only stimulate proliferation in B cells whose slg had not been modulated during the 2-hr preculture period. In contrast, higher concentrations of anti-δ-dex antibody that

Table 2. B-cell stimulation induced by anti-δ-dex does not correlate with its ability to induce receptor modulation, increases in [Ca2+]i, or increases in PIP2 breakdown

<table>
<thead>
<tr>
<th>Stimulus, ( \mu g/ml )</th>
<th>Mean max ([Ca^{2+}]i, \text{nM} )</th>
<th>Mean max (\text{in} )</th>
<th>Mean max (\text{phosphates, cpm} )</th>
<th>Mean max (\text{CSA} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>150</td>
<td>98</td>
<td>24,000</td>
<td>12,000</td>
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<tr>
<td>anti-δ-dex</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>1</td>
<td>848</td>
<td>1110</td>
<td>1,600</td>
<td>31,400</td>
</tr>
<tr>
<td>10(^{-1})</td>
<td>668</td>
<td>606</td>
<td>10,300</td>
<td>32,000</td>
</tr>
<tr>
<td>10(^{-2})</td>
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<td>134</td>
<td>12,700</td>
<td>31,300</td>
</tr>
<tr>
<td>10(^{-3})</td>
<td>196</td>
<td>78</td>
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<td>10(^{-4})</td>
<td>157</td>
<td>83</td>
<td>24,700</td>
<td>26,300</td>
</tr>
<tr>
<td>10(^{-5})</td>
<td>146</td>
<td>ND</td>
<td>25,000</td>
<td>21,000</td>
</tr>
<tr>
<td>anti-δ</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>100</td>
<td>393</td>
<td>346</td>
<td>1,000</td>
<td>31,700</td>
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<td>194</td>
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<tr>
<td>10(^{-3})</td>
<td>143</td>
<td>ND</td>
<td>22,400</td>
<td>12,600</td>
</tr>
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</table>

Resting B lymphocytes were placed in culture at 10⁶ per ml in the presence of indicated concentrations of anti-δ-dex or anti-δ + 10 µg of 24G2 per ml. Results are expressed as channel scale area (CSA), the sum of the products of cell number and their staining intensity. Results are compared to mean max. \([Ca^{2+}]i\) levels were obtained from cells treated as described in the legend to Fig. 1. Mean max is the mean of four consecutive channels of data that attained highest levels of \([Ca^{2+}]i\), in a 16-sec period during which ~500 cells per sec were analyzed. Parallel cultures were analyzed for PIP2 hydrolysis. Results presented are representative of five different experiments that gave similar results. ND, not determined.
were both stimulatory and effective modulators of slg would stimulate proliferation of B cells even when slg had already been modulated. This proved to be the case, in that B cells whose slg had been modulated by prior exposure to the nonmitogenic anti-δ antibody responded without any significant diminution to the higher concentrations (10^{-1} and 10^{-2} μg/ml) but not to the lower concentrations (10^{-3} or 10^{-4} μg/ml) of anti-δ-dex antibody (Fig. 3A). This supports the view that the low nonmodulatory concentrations of anti-δ-dex antibody are mitogenic only when encountering B cells on which slg molecules are not reduced in number and so may transmit repetitive and prolonged signal transduction. To address the issue of repetitive signal transduction directly we employed purified IgD to inhibit competitively the binding of anti-δ-dex to slgD and thereby serve to interrupt continuous signal transduction. IgD was added at a 50,000-fold molar excess at the start of culture as well as at 6 and 24 hr after the initiation of a 48-hr culture (Fig. 3B). Addition of IgD as late as 24 hr still had a significant suppressive effect (62% suppression) on anti-δ-dex-stimulated B-cell proliferation. Taken together, these two experiments suggest that optimal stimulation of B-cell DNA synthesis by low concentrations of anti-δ-dex requires prolonged and repetitive signaling. Such cells that undergo persistent signal transduction may not require the rapid and high-level increases in [Ca^{2+}]; to initiate or maintain their activation cycle as is required by cells stimulated by higher concentrations of unconjugated anti-Ig, which rapidly modulates slg. Rather, in such cells comparable biochemical events may occur at later time points, and at very low levels, after the initiation of cell activation and could persist over longer periods. Alternatively, low levels of calcium transients may be induced at the plasma membrane that may not be reflected in measurements of cytoplasmic [Ca^{2+}]. Such a mode of B-cell activation may therefore not require the additional stimulation provided by T cells or T-cell-derived lymphokines and therefore can be considered to be relatively T-cell independent.

Other interpretations of these results that cannot be excluded by these data include these possibilities: (i) Very low concentrations of multimeric antigens or anti-δ-dex may recruit inositol phospholipid-independent pathways of activation as is stimulated by the polyclonal B-cell-activator lipopolysaccharide (15) and recently also shown to be stimulated by ligation of the T-cell-antigen receptor (24, 25). This possibility is also consistent with our previous observation that anti-Ig can deliver stimulatory signals to B cells depleted of PKC (26). (i) The mitogenic effect of even low concentrations of anti-δ-dex is dependent upon its capacity to induce individual B cells to experience recurrent, transient increases in [Ca^{2+}]; (20, 27). In view of the finding of Wilson et al. (20) that anti-δ induces oscillations in [Ca^{2+}], that persist for 15–60 sec and the observation that our system is sensitive to changes in the level of [Ca^{2+}] in <1% of cells that last for <1 sec, we view this possibility as unlikely.

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