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Immunology

Antigen-independent regulation of cytoplasmic calcium in B cells with a 12-kDa B-cell growth factor and anti-CD19

(B-cell activation/signal transduction/indo-1/leukemic progenitor cells)

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ABSTRACT **Increases in cytoplasmic free calcium ($[Ca^{2+}]_i$) can be induced in resting B cells either by a low molecular weight (12-kDa) B-cell growth factor (LMW-BCGF) or by crosslinking the B-cell antigen CD19 with monoclonal antibody (mAb). LMW-BCGF causes a slow $[Ca^{2+}]_i$ increase in peripheral blood and tonsillar B cells but has no effect on $[Ca^{2+}]_i$ in resting T cells. B-cell $[Ca^{2+}]_i$ responses mediated by anti-surface immunoglobulin (sIg) or anti-CD19 are potentiated by LMW-BCGF, but anti-sIg and anti-CD19 do not show additive $[Ca^{2+}]_i$ responses. LMW-BCGF- and anti-CD19-induced $[Ca^{2+}]_i$ signals are similar to the sIgM or sIgD-mediated signals in that they are inhibited by prior treatment with phorbol 12-myristate 13-acetate. However, LMW-BCGF- and CD19-mediated signals do not depend on the expression of sIg, since they were also observed on sIg- B-cell precursor acute lymphoblastic leukemia (ALL) cells. Both anti-CD19 and LMW-BCGF stimulated *in vitro* colony formation by ALL cells and showed additive effects when used together. $[Ca^{2+}]_i$ responses to LMW-BCGF or CD19 crosslinking were also evident on certain pre-B-cell and lymphoma B-cell lines.**

Antigen-specific activation of resting B cells occurs via signals transmitted by crosslinking of surface immunoglobulins (sIg). This activation is mediated by increased inositolphospholipid metabolism. The resulting production of diacylglycerol and inositol 1,4,5-trisphosphate ($InsP_3$) leads to activation of protein kinase C and elevation of cytoplasmic calcium concentration ($[Ca^{2+}]_i$) (1–3). However, a second signal, complementary to the sIg-mediated signal, is required to drive resting B cells into S phase (4–6). The complementary or "progression" signal can be provided by a variety of growth factors (γ interferon, interleukins 1, 2, and 4, 12- and 60-kDa B-cell growth factors), complement components (C3d), or monoclonal antibodies (mAbs) binding to B-cell receptors (anti-CDw40, anti-CD23, and anti-CD22) (7–20). In general, the signals for B cells mediated by these factors and antibodies have not been biochemically identified. One of these factors, 12-kDa or low molecular weight B-cell growth factor (LMW-BCGF), produced by lectin-activated T cells, is able to support clonal B-cell proliferation and is a predominant factor affecting B cells (12, 21).

Signals that inhibit sIg-mediated B-cell activation have also been identified. One of these, the B-cell Fc region receptor, appears to inhibit inositolphospholipid metabolism (22). Antibody binding to the CD19 B-cell receptor also blocks B-cell proliferation and B-cell $[Ca^{2+}]_i$ responses to anti-immunoglobulin (23, 24). CD19 crosslinking alone can stimulate a $[Ca^{2+}]_i$ response in resting B cells without leading to B-cell activation (23, 24).

In this report we examine the effects of LMW-BCGF and CD19 ligation on $[Ca^{2+}]_i$ responses in B cells at different stages of maturation. LMW-BCGF increased $[Ca^{2+}]_i$ in resting B cells and augmented the $[Ca^{2+}]_i$ response to anti-sIg or CD19 crosslinking. The effect was specific, since other growth factors did not affect B-cell $[Ca^{2+}]_i$ levels. The activity of LMW-BCGF or CD19 crosslinking did not depend on expression of sIg, since some sIg^- pre-B-cell acute lymphoblastic leukemias (ALLs) showed $[Ca^{2+}]_i$ signals in response to LMW-BCGF or anti-CD19. In addition, we found that CD19 stimulation caused B-cell precursor ALL cells to proliferate and form colonies *in vitro*. This suggests that the CD19 receptor can function as either a positive or a negative signal for proliferation, depending upon the stage of B-cell differentation and activation.

MATERIALS AND METHODS

mAbs and Reagents. mAbs B43, 2H7, and 9.6 against human antigens CD19, CD20, and CD2, respectively, have been described (24–26). The $F(ab')_2$ fragment of goat antibodies against human μ chain was obtained from Jackson Research Labs (West Grove, PA), and δTA4-1 anti-human δ chain hybridoma cell line (27) was obtained from the American Type Culture Collection. Rat mAb 187.1 against mouse κ chain (28) was purified as described (26). mAb BA-5 against the receptor for a high molecular weight (90-kDa) B-cell growth factor (HMW-BCGF) was provided by Julian Ambrus (National Institutes of Health, Bethesda, MD). EGTA and phorbol 12-myristate 12-acetate (PMA) were from Sigma. Recombinant interleukin 2 (rIL-2) was purchased from Genzyme (Norwalk, CT) and recombinant interleukin 1β (rIL-1β, $>10^8$ units/mg) (29) was provided by S. Gillis (Immunex, Seattle). Recombinant human interleukin 4 (rIL-4) (11) was provided by J. Bonchereau (UNICET, Pardilly, France). Recombinant granulocyte colony-stimulating factor (rG-CSF, $>10^6$ units/mg) (30) was provided by L. Souza (Amgen, Thousand Oaks, CA). Recombinant interleukin 3 (rIL-3) (31) and recombinant granulocyte–macrophage colony-stimulating factor (rGM-CSF,

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Abbreviations: $[Ca^{2+}]_i$, cytoplasmic calcium concentration; mAb, monoclonal antibody; PMA, phorbol 12-myristate 13-acetate; LMW-BCGF, low molecular weight B-cell growth factor; HMW-BCGF, high molecular weight B-cell growth factor; rIL-1β, recombinant interleukin 1β; rIL-4, recombinant interleukin 4; rG-CSF, recombinant granulocyte colony-stimulating factor; rGM-CSF, recombinant granulocyte–macrophage colony-stimulating factor; rIL-3, recombinant interleukin 3; rIL-2, recombinant interleukin 2, sIg, surface immunoglobulin(s); ALL, acute lymphoblastic leukemia; $InsP_3$, inositol 1,4,5-trisphosphate; PHA, phytohemagglutinin; PE, phycoerythrin.

>10^6 units/mg) (32) were provided by S. Clark (Genetics Institute, Cambridge, MA). Biologically active natural LMW-BCGF (12 kDa) was prepared from a pooled phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cell conditioned medium by using a series of column chromatographic steps including preparative-scale DEAE-cellulose chromatography, hydroxyapatite column chromatography, ion-exchange, and size exclusion HPLC (33). The specific activity of biochemically purified LMW-BCGF was estimated to be 10^6 units/mg of protein by Bio-Rad assays. LMW-BCGF (12-kDa) preparations did not contain detectable amounts of IL-1, IL-2, IL-3, GM-CSF, G-CSF, or γ interferon as determined by standard bioassays, but they were able to induce proliferation of sIg-activated B cells, with a maximal stimulation index of 23.6. Furthermore, we have ruled out the possibility that residual contaminating PHA is responsible for any of the $[Ca^{2+}]_i$ activity described here, since an anti-PHA mAb, G26-5, was able to inhibit 94% of the PHA-mediated $[Ca^{2+}]_i$ increase in B cells without any effect on the $[Ca^{2+}]_i$ signal from LMW-BCGF (data not shown).

Measurement of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was measured with the dye indo-1 (Molecular Probes, Junction City, OR) and a model 50HH/2150 cell sorter (Ortho Diagnostics, Raritan, NJ) as we have described in detail (34). Peripheral blood from normal donors or tonsillar lymphocytes were isolated by centrifugation on Ficoll/Hypaque before loading with the indo-1 acetoxymethyl ester (34). B cells were subsequently analyzed by fluorescence gating on $CD20^+$ cells after staining with phycoerythrin (PE)-conjugated mAb 2H7 (20). Light-scatter gating was used to analyze single cells, eliminating signals potentially related to cell–cell contact.

B-Cell Precursor Colony Assay. Leukemic B-cell precursors from patients were assayed for colony formation *in vitro* by using a B-cell precursor colony assay system as previously described (35, 36). Leukemic B-cell precursor blasts were suspended in α minimal essential medium (GIBCO) supplemented with 0.9% methylcellulose, 30% calf serum, 1% penicillin/streptomycin, and various concentrations of LMW-BCGF, B43 (anti-CD19), and BA-5 (anti-HMW-BCGF receptor). Triplicate 1-ml samples containing 10^5 blasts were cultured in 35-mm Petri dishes for 7 days at 37°C in a humidified 5% CO_2 atmosphere. On day 7, colonies containing >20 cells were counted on a grid by using an inverted phase-contrast microscope with high optical resolution. Subsequently, colonies were harvested, and morphological, immunological, and cytogenetic features of colony cells were analyzed as described (36).

RESULTS

LMW-BCGF Increases $[Ca^{2+}]$ in B Cells. Growth factors such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) exert their biological activity partially by increasing $[Ca^{2+}]_i$ (37). We therefore tested LMW-BCGF and several other factors to determine the effect on $[Ca^{2+}]_i$ in lymphocytes. Peripheral blood B cells, identified by fluorescence staining for the CD20 antigen (Fig. 1*A*) exhibit an increased $[Ca^{2+}]_i$ 30 min after LMW-BCGF addition (Fig. 1*B*), whereas at the same time non-B cells ($CD20^-$) had $[Ca^{2+}]_i$ identical to that of control cells (Fig. 1*C*). A similar B-cell specific response to LMW-BCGF was seen with $CD2^-$ (non-T) tonsillar cells, indicating that the $[Ca^{2+}]_i$ response was not dependent upon the binding of the CD20 mAb 2H7 (Fig. 1 *D–F*). The effect on B cells was not apparent with other growth factors such as rG-CSF (50 ng/ml) or rIL-2 (1000 units/ml) (Fig. 1). Furthermore, there was no effect on $[Ca^{2+}]_i$ in resting T or B cells after addition of rIL-4 (1000 units/ml), rIL-1β (10 ng/ml), rIL-3 (10 units/ml), or rGM-CSF (50 ng/ml) (data not shown).

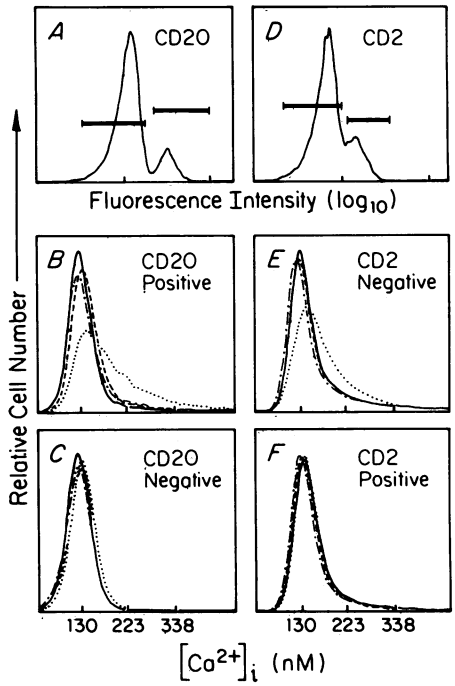

FIG. 1. $[Ca^{2+}]_i$ responses to LMW-BCGF by peripheral blood (*A–C*) and tonsillar (*D–F*) B cells. (*A*) Peripheral blood B cells were identified by staining with PE-conjugated mAb 2H7 (anti-CD20). Cells were gated as indicated by the horizontal bars. Cells were stimulated with medium (—), rIL-2 at 1000 units/ml (----), rG-CSF at 50 ng/ml (·---), or LMW-BCGF at 2 ng/ml (·····). $CD20^+$ B cells (*B*; the right-hand gate in *A*) and $CD20^-$ cells (*C*; the left-hand gate in *A*) were simultaneously analyzed for their $[Ca^{2+}]_i$ response 30 min after stimulation. (*D*) Tonsillar T lymphocytes were identified by staining with PE-conjugated mAb 9.6 (anti-CD2). $CD2^-$ (*E*) and $CD2^+$ (*F*) cells were simultaneously analyzed for their $[Ca^{2+}]_i$ response 30 min after stimulation with the same growth factors.

The kinetics and magnitude of the $[Ca^{2+}]_i$ response to LMW-BCGF by resting tonsillar B cells are shown in Fig. 2. The onset of the response was delayed, and the response required 10–15 min to reach a maximum. The 2 ng/ml of LMW-BCGF used in this experiment corresponded to approximately 2 units/ml and was able to give a detectable signal, indicating that the $[Ca^{2+}]_i$ increase and the functional activity of LMW-BCGF occurred at similar doses. When higher levels of LMW-BCGF were tested (10 ng/ml), a slight

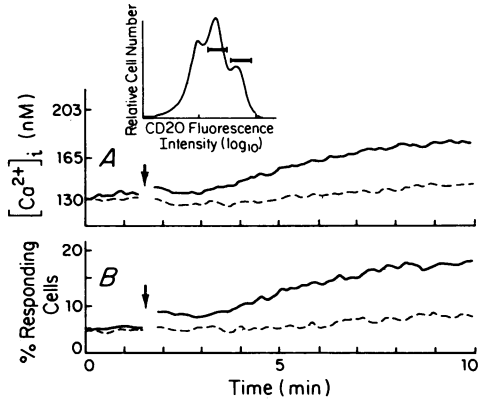

FIG. 2. Response to LMW-BCGF by a subpopulation of tonsillar B cells. (*Inset*) Tonsil lymphocytes were stained with PE-conjugated mAb 2H7 (anti-CD20). CD20-bright B cells (------) and CD20-dull B cells (——) were simultaneously analyzed after stimulation at 1.5 min with LMW-BCGF at 2 ng/ml. The $[Ca^{2+}]_i$ response (*A*) and the percent of cells responding (*B*) are shown.

increase in velocity of response was seen (data not shown). The experiment (Fig. 2) utilized tonsillar B cells stained with anti-CD20. The "CD20-dull" B cells correspond to dense, resting, IgM^+ B cells predominantly from mantle zone regions of secondary follicles, whereas the "CD20-bright" B cells correspond to buoyant, activated, IgM^- B cells predominantly from germinal center regions (25). The $[Ca^{2+}]_i$ response to KMW-BCGF occurred among CD20-dull resting B cells, whereas no response occurred among the CD20-bright activated B cells (Fig. 2*A*). In other experiments, we have seen that CD20-bright B cells also fail to respond in $[Ca^{2+}]_i$ assays to anti-immunoglobulin or anti-CD19 stimulation (data not shown). Notably, only about 15–20% of CD20-dull tonsillar B cells responded with a $[Ca^{2+}]_i$ increase greater than two standard deviations above the mean $[Ca^{2+}]_i$ of resting B cells (Fig. 2*B*). However, this probably underestimates the number of responding cells, since the small $[Ca^{2+}]_i$ signal does not drive all responding B cells above the threshold.

The $[Ca^{2+}]_i$ response in B cells after stimulation with an optimal dose of anti-μ occurred very rapidly, as previously noted (3). When LMW-BCGF was added simultaneously with anti-μ, the $[Ca^{2+}]_i$ response during the initial phase was not altered, but the late response, 5–10 min after stimulation, was maintained at a higher level (Fig. 3*A*). CD19 stimulation, which requires crosslinking with a secondary antibody to generate a maximal $[Ca^{2+}]_i$ signal (23), was similarly augmented with LMW-BCGF (Fig. 3*B*). However, the CD19 signal and the anti-μ signal were not additive in their ability to increase $[Ca^{2+}]_i$ in B cells (Fig. 3). The secondary antibody used was 187.1, a purified rat mAb against mouse κ chain that by itself had no effect on $[Ca^{2+}]_i$, (data not shown). The CD19 signal from crosslinking mAb B43 (10 μg) with 40 μg of mAb 187.1 generated a maximal $[Ca^{2+}]_i$ response. Similarly, the signal from anti-μ was maximal with 10 μg/ml, and 187.1 had no effect on the anti-μ signal (data not shown).

Effect of PMA on $[Ca^{2+}]_i$ Signals in B Cells. Prior treatment with PMA has been reported to inhibit $[Ca^{2+}]_i$ signaling after anti-immunoglobulin stimulation in B cells (38, 39). We therefore examined the effect of PMA on CD19 and LMW-BCGF-stimulated $[Ca^{2+}]_i$ responses. Both anti-CD19- and LMW-BCGF-driven responses were similar to anti-μ and anti-δ responses in that they were inhibited in cells after a 10-min prior treatment with PMA (Fig. 4). Since PMA is a direct activator of protein kinase C, these results suggest that CD19, LMW-BCGF, and anti-immunoglobulin-mediated $[Ca^{2+}]_i$ signals are similar in that they are all influenced by protein kinase activation, possibly by relying on an intermediate regulated by this enzyme.

CD19 and LMW-BCGF Stimulate Proliferation and Increase $[Ca^{2+}]_i$ of B-Lineage Cells at Multiple Stages of Differentiation. To evaluate the effect of LMW-BCGF and CD19 ligation on $[Ca^{2+}]_i$ as well as proliferation at very early differentiation stages within the B-cell precursor pathway, we used leukemic cells from B-cell precursor ALL patients. These early B-lineage cells display a surface antigen profile (sIg^- Cu^- TdT^+ $CD10^+$ $CD19^+$ $CD24^+$; Cu is cytoplasmic μ chain and TdT is terminal deoxynucleotidyltransferase) consistent with a maturation arrest at the B-cell precursor stage. In some experiments, B-cell precursors showed a strong and rapid $[Ca^{2+}]_i$ response to BCGF, as evidenced by one representative case depicted in Fig. 5*A*. Notably, besides LMW-BCGF, anti-CD19 mAb B43 also induced a $[Ca^{2+}]_i$ increase that was further augmented by crosslinking with the anti-mouse κ secondary mAb 187.1 (Fig. 5*A*). In contrast to LMW-BCGF or mAb B43, neither rIL-1β nor rIL-2 affected $[Ca^{2+}]_i$ levels. A second example is shown in Fig. 5*B*. A strong response to CD19 was seen that depended completely upon the secondary mAb for crosslinking, but LMW-BCGF generated no detectable response. In addition to the very immature B-cell precursors (pre-pre-B stage), we also tested a number of B-lineage cell lines at later stages of differentiation for their $[Ca^{2+}]_i$ responses to LMW-BCGF and CD19 crosslinking. As depicted in Fig. 5*C*, the sIg^- Cu^+ TdT^+ $CD10^+$ $CD19^+$ (pre-B stage) NALM-6 cell line responded to CD19 mAb, but no detectable response to LMW-BCGF was evident. However, the response to CD19 was increased by the simultaneous addition of CD19 and

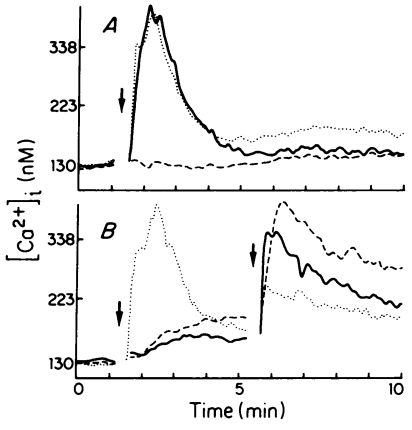

FIG. 3. LMW-BCGF augments $[Ca^{2+}]_i$ response to anti-μ or CD19 stimulation of peripheral blood B cells. Peripheral blood B cells were identified by staining with PE-conjugated mAb 2H7 (anti-CD20) as described for Fig. 1. (*A*) Responses of $CD20^+$ cells to stimulation at 1.5 min with $F(ab')_2$ anti-μ at 10 μg/ml (——), LMW-BCGF at 2 ng/ml (------), or both together (·····). (*B*) Response of $CD20^+$ cells to stimulation with mAb B43 (anti-CD19) at 10 μg/ml followed by addition of mAb 187.1 (anti-mouse κ) at 40 μg/ml at the second arrow (5.5 min) (——), response to simultaneous addition of LMW-BCGF at 2 ng/ml plus mAb B43 at 10 μg/ml at the first arrow (1.5 min) followed by mAb 187.1 at 40 μg/ml at the second arrow (------), and response to simultaneous addition of mAb B43 at 10 μg/ml plus $F(ab')_2$ anti-μ at 10 μg/ml at the first arrow followed by mAb 187.1 at 40 μg/ml at the second arrow (·····).

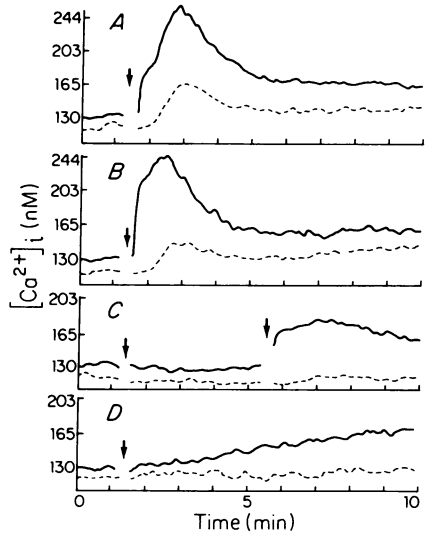

FIG. 4. Effect of prior treatment with PMA on $[Ca^{2+}]_i$ signals from CD19 and LMW-BCGF. Tonsillar lymphocytes were treated with PMA at 100 ng/ml for 10 min prior to stimulation (------); control lymphocytes were not treated with PMA (——). (*A*) $[Ca^{2+}]_i$ response to goat anti-μ $F(ab')_2$ at 10 μg/ml. (*B*) Response to anti-δ (TA4-1) at 5 μg/ml. (*C*) Response to CD19 stimulation (mAb B43 at 10 μg/ml) at 1.5 min followed by mAb 187.1 at 40 μg/ml at 5.5 min. (*D*) Response to LMW-BCGF (2 ng/ml).

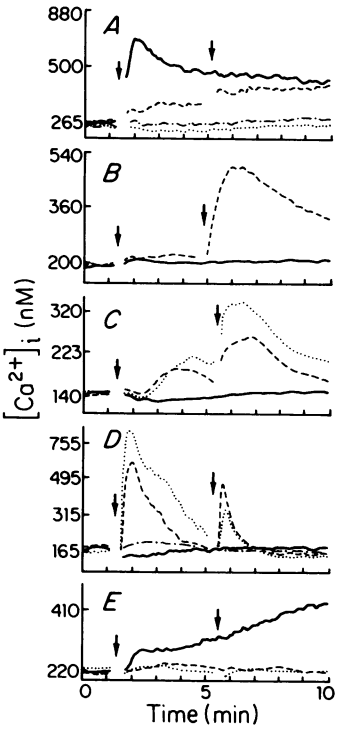

FIG. 5. Response of B-cell ALLs and B-cell lines to stimulation with LMW-BCGF or anti-CD19. (*A*) B-cell ALL stimulated with LMW-BCGF at 2 ng/ml (——), B43 at 10 μg/ml at 1.5 min plus 187.1 at 40 μg/ml at 5.5 min (------), rIL-2 at 200 units/ml (.....), or rIL1-β at 10 ng/ml (-·-·-·). (*B*) ALL no. 2 stimulated with 10% (vol/vol) LMW-BCGF (——) or B43 at 10 μg/ml at 1.5 min followed by 187.1 at 40 μg/ml at 5.5 min (------). (*C*) NALM-6 pre-B-cell line stimulated with LMW-BCGF at 2 ng/ml (——), B43 at 10 μg/ml at 1.5 min followed by 187.1 at 40 μg/ml at 5.5 min (------), or LMW-BCGF at 2 ng/ml plus B43 at 10 μg/ml at 1.5 min followed by 187.1 at 40 μg/ml at 5.5 min (.....). (*D*) DHL-10 lymphoma cell line stimulated with LMW-BCGF at 2 ng/ml (——), goat anti-μ $F(ab')_2$ at 10 μg/ml (-·-·-·), B43 at 10 μg/ml at 1.5 min followed by 187.1 at 40 μg/ml at 5.5 min (-----), or LMW-BCGF at 2 ng/ml plus B43 at 10 μg/ml at 1.5 min followed by 187.1 at 40 μg/ml at 5.5 min (.....). (*E*) P3HR1 lymphoma cell line stimulated with LMW-BCGF at 2 ng/ml (——), goat anti-μ $F(ab')_2$ at 10 μg/ml (------), or B43 at 10 μg/ml at 1.5 min followed by 187.1 at 40 μg/ml at 5.5 min (.....).

LMW-BCGF. DHL-10 is a TdT^- $CD10^-$ $CD19^+$ sIg^+ (immature B-immunoblast stage) non-Hodgkins lymphoma cell line. DHL-10 cells did not respond to anti-μ stimulation even though they are sIg^+ (Fig. 5*D*). DHL-10 cells did respond to CD19 stimulation, and their response to CD19 ligation was augmented by LMW-BCGF. P3HR1 is a TdT^- $CD10^-$ $CD19^+$ sIg^+ (mature B-immunoblast stage) Burkitt lymphoma that responded well to LMW-BCGF, exhibiting a 200 nM increase in $[Ca^{2+}]_i$, but showed no detectable response to CD19 or anti-μ (Fig. 5*E*). These findings provide circumstantial evidence that different signal transmission pathways may be operative at distinct stages of B-cell development.

A small fraction of B-cell precursors in leukemic marrow samples from B-cell precursor ALL patients are B-lineage lymphoid progenitor cells that represent the earliest detectable B-lineage cells in the human hematopoietic system. Importantly, B-lineage lymphoid progenitor cells form B-cell precursor colonies in an *in vitro* assay system that was recently described (24, 33, 35, 36). When tested for biological effects on B-lineage lymphoid progenitor cells in 17 cases (Table 1), anti-CD19 mAb B43 induced proliferation and B-cell precursor colony formation in 3 cases and augmented spontaneous colony formation in 4 cases. Hence, while

Table 1. Anti-CD19 mAb B43 augments LMW-BCGF-stimulated formation of B-cell precursor colonies *in vitro*

Factor	mAb added	mAb conc., μg/ml	No. of cases with colony formation	No. of B-cell precursor colonies per 10^5 cells	
				Mean	Range
None	None	—	4/17	55	15–105
None	B43	10	7/13	211	70–416
LMW-BCGF	None	—	14/17	775	14–2300
LMW-BCGF	B43	0.1	5/9	1116	352–2286
LMW-BCGF	B43	1.0	5/8	1281	403–2794
LMW-BCGF	B43	5.0	5/8	1473	498–2985
LMW-BCGF	B43	10.0	14/17	1091	24–2804

Highly blast-enriched fresh B-cell precursor ALL marrow samples were cultured in the presence of purified LMW-BCGF (2 ng/ml) and affinity-purified mAb B43 at various concentrations and were assayed for blast colony formation as described (33, 35, 36). Results are shown as the mean number of B-cell precursor ALL blast colonies per 1×10^5 cells cultured. The variations in colony number between replicate samples did not exceed 5% of the mean values.

CD19 mAb B43 inhibits proliferation of mature B cells in response to anti-μ stimulation, it provides a positive proliferative signal for the most immune B-cell precursors. Table 1 also illustrates that in 14 of 17 cases, B-lineage lymphoid progenitor cells showed a marked proliferative response to LMW-BCGF. The mean number of B-cell precursor colonies was 211 in cultures stimulated with CD19 mAb B43 (range 70–416) and 775 (range 14–2300) in cultures stimulated with LMW-BCGF. Notably, the combination of B43 plus LMW-BCGF provided a stronger proliferative signal for B-lineage lymphoid progenitor cells than B43 alone or LMW-BCGF alone. The observed potentiation of BCGF effects was specific for B43, since the control mAbs BA-5 and BA-1 did not augment the proliferative response of B-lineage lymphoid progenitor cells to LMW-BCGF (data not shown).

Detailed immunological analyses on B-cell precursor colony cells were performed to determine whether the LMW-BCGF or anti-CD19 mAb B43 signals induce differentiation in B-cell precursors. Colony cells were TdT^+, $CD10^+$, $CD19^+$, $CD20^-$, CD22 (cytoplasmic)$^+$, and $CD24^+$, but lacked sIg and cytoplasmic μ heavy chains (data not shown). Thus neither LMW-BCGF nor mAb B43 induces further differentiation in leukemic B-cell precursors.

DISCUSSION

The present study shows that purified (12-kDa) LMW-BCGF causes an increase in $[Ca^{2+}]_i$ that is specific for resting B cells in peripheral blood and tonsils, indicating that resting B cells have functional receptors for LMW-BCGF. The $[Ca^{2+}]_i$ response was relatively slow, taking at least 10 min to reach a maximum that represented an increase of 40–50 nM $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ response to anti-δ or anti-μ stimulation occurred with more rapid kinetics and reached a much higher peak, which was not sustained. When LMW-BCGF and anti-μ were used simultaneously, the $[Ca^{2+}]_i$ response was sustained at higher levels than with the anti-μ alone. This is consistent with the established ability of LMW-BCGF to cooperate with stimulation of sIg to drive resting B cells through the cell cycle. Although CD20-bright B cells in tonsils did not respond to LMW-BCGF with a detectable increase in $[Ca^{2+}]_i$, these cells also did not respond to CD19 stimulation. Separate experiments will be required to determine whether resting B cells activated *in vitro* will respond to LMW-BCGF with a $[Ca^{2+}]_i$ increase.

The increased $[Ca^{2+}]_i$ that occurs after sIg stimulation of B cells is initiated by the phospholipase C-mediated production of $InsP_3$ and subsequent release of cytoplasmic stores of calcium (1–3). In murine B cells and B-cell lines, the $InsP_3$ production and $[Ca^{2+}]_i$ response are inhibited by prior treatment with PMA, a direct activator of protein kinase C (38, 39). It was postulated that protein kinase C phosphorylates a protein that is critical to the sIg-mediated signal transduction in B cells (38, 39). Our results demonstrate that $[Ca^{2+}]_i$ responses to anti-immunoglobulin by human B cells are also inhibited by prior treatment with PMA. The LMW-BCGF- and CD19-mediated $[Ca^{2+}]_i$ responses were also sensitive to PMA, suggesting that these signals are also mediated by phospholipase C activation that results in $InsP_3$ formation. In the case of CD19, our previous results showing that a portion of the $[Ca^{2+}]_i$ signal is independent of extracellular calcium supports this view (23).

sIg^-, Cu^- leukemic B cell precursors can respond to LMW-BCGF by colony formation in an *in vitro* assay (23). In addition, CD19, which was previously shown to regulate $[Ca^{2+}]_i$ and inhibit sIg signalling in normal B cells (23, 24), is expressed in high surface density on B-cell precursors. We therefore tested fresh leukemic B cell precursor blasts and B-lineage cell lines for their responsiveness to CD19 and LMW-BCGF in $[Ca^{2+}]_i$ and colony formation assays. Our data show that many of these sIg^- cells are responsive to CD19 and LMW-BCGF in both assays. In addition, we found that cell lines NALM-6 (pre-B, sIg^-, Cu^+; Fig. 5) and REH (pre-pre-B, sIg^-, Cu^-; data not shown) showed $[Ca^{2+}]_i$ responses to CD19 crosslinking that were augmented by LMW-BCGF. Thus the receptors for CD19 and LMW-BCGF do not depend upon the expression of sIg to transmit their signals.

The observed effects on $[Ca^{2+}]_i$ appear to be specific for LMW-BCGF, since we found no activity with rIL-1β, rIL-2, rIL-3, rIL-4, rGM-CSF, or rG-CSF. Furthermore, the activity of LMW-BCGF was B-cell specific in that resting B cells but not other cells responded with an increase in $[Ca^{2+}]_i$ (Fig. 1). Recent evidence has suggested that the CD23 B-cell-specific antigen may be a receptor for LMW-BCGF, since anti-CD23 mAbs mimic the effects of LMW-BCGF in functional experiments and block the binding of LMW-BCGF to B cells (18, 40). Thus it will be important to determine the relationship between the CD23 antigen and the LMW-BCGF $[Ca^{2+}]_i$ signal.

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