Brief Definitive Report

Immunoglobulin-mediated Signal Transduction in B Cells from CD45-deficient Mice

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Summary

CD45 expression is essential for immunoglobulin (Ig)-mediated B cell activation. Treatments with either anti-Ig or anti-CD45 suggest that CD45 may facilitate early signaling events such as calcium mobilization, and phosphoinositide hydrolyis as well as later events leading to transcription of genes such as c-*myc*. To examine the role of CD45 more extensively, CD45-deficient mice were generated by disruption of exon 6. Although normal numbers of B cells were found in peripheral lymphoid tissues, CD45-deficient cells failed to proliferate upon IgM cross-linking. In the present study, we demonstrate that the fraction of high buoyant density B cells is reduced while low buoyant density cells are increased. Moreover, there is a significant decline in the number of splenic B cells of the mature IgD^{hi}, IgM^{lo} phenotype. Although both the basal and anti-Ig–induced levels of phosphorylation of Ig-α and phospholipase Cγ2 are indistinguishable from that observed in $CD45^+$ control B cells, a major distinction was found in Ca^{2+} mobilization. While anti-Ig–induced mobilization of intracellular Ca^{2+} stores was normal, influx from extracellular sources was abrogated. This finding reveals a novel pathway of regulating B cell responses mediated by CD45.

Evidence from a variety of sources clearly demonstrates that the expression of CD45 is essential for activation of B lymphocytes after cross-linking of the B cell receptor (BCR) (1–6). In vitro activation studies using primary cells treated with either anti-Ig or anti-CD45 suggest that CD45 facilitates early signaling events such as calcium mobilization (6), and phosphoinositide hydrolyis as well as later events leading to transcription of genes such as c-*myc* (7). Cell lines have also been used to examine the role of CD45. In some cases conflicting results have emerged from such studies. For example, it was demonstrated that the plasmacytoma J558Lμm3, which lacks CD45, failed to mobilize Ca^{2+} after BCR cross-linking. This effect was restored in J558Lμm3 cells by transfection with a cDNA encoding CD45 (1). In contrast, $CD45^-$ variants of the $CD45^+$ immature cell line WEHI231, had a slightly delayed but increased and prolonged Ca^{2+} response upon BCR cross-linking (8). Whether this discrepancy accurately reflects the different maturation states of these cell lines, or is due to abnormalities peculiar to transformed cells, remains to be determined.

To examine the role of CD45 more extensively, CD45-deficient mice were generated by disruption of exon 6 (9). Mice homozygous for the CD45 exon 6 mutation lacked CD45 expression on B cells and on the majority of thymocytes and peripheral T cells. A block in T cell development occurred at the transition from immature $CD4^+CD8^+$ double-positive to mature single-positive thymocytes resulting in a significant reduction in peripheral T cells. Residual peripheral T cells exhibited impaired anti-CD3 and anti-TCR signaling. In contrast to the paucity of peripheral T cells, approximately normal numbers of B cells were found in peripheral lymphoid tissues. Furthermore, the B cells detected were responsive to the B cell mitogen, lipopolysaccharide, and serum levels of both IgM and IgG were near normal. However, thymidine incorporation assays revealed that CD45-deficient cells failed to proliferate after cross-linking with IgM-specific antibodies. These results confirmed that CD45 plays a critical role in B cell activation as previously demonstrated in other experimental systems.

Materials and Methods

Mice. Mice used were CD45 exon 6 −/−, originally derived by Kenji Kishihara, after fifth backcross with C57BL/6. Control mice were either age-matched C57BL/6 or CD45 exon 6 +/+ mice of the fifth backcross to C57BL/6.

B Cell Purification. Bone marrow was prepared by flushing tibias and femurs with a 26-gauge syringe. Single cell suspensions of splenic B cells were prepared by mincing over a metal screen with a syringe plunger followed by a 5-min incubation on ice to remove large aggregates. Erythrocytes were lysed with ACK (0.155 M ammonium chloride, 0.1 mM disodium EDTA, 0.01 M potassium bicarbonate, pH 7.3) for 5 min on ice. T cells were

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lysed with anti-CD4 (RL172), anti-CD8 (3.168), and anti-thy1.2 (HO13.4) antibody supernatants (1:20 dilution) together with low tox guinea pig complement (1/12 dilution) (Cedarlane Laboratories Ltd., Hornby, Canada) for 1 h at 37°C. The remaining cells were separated on a Percoll gradient (Sigma Chemical Co., St. Louis, MO) as described (10).

[³H]Thymidine Incorporation Assay. Splenic B cells were placed in flat-bottomed 96-well plates (Costar Corp., Cambridge, MA) in OPTI-MEM (GIBCO BRL, Gaithersburg, MD) medium supplemented with 2.4 g/liter $NaHCO_3$, 5 mg/ml streptomycin, 5×10^3 U/liter penicillin, 5×10^{-5} M 2-mercaptoethanol, 0.5% FCS (GIBCO BRL, Gaithersburg, MD) at 10^5 cells/well with either medium alone, LPS (20 μg/ml), B76 (rat IgG1 anti–mouse Igμ; 30 μg/ml), or goat anti–mouse Igμ (30 μg/ml; Jackson Immunoresearch Laboratories, West Grove, PA) at various concentrations. B cells were harvested at various intervals after a 6-h pulse with 1 μCi of [³H]thymidine per well.

Flow Cytometry. Cells ($1–5 \times 10^5$/sample) were washed and exposed to appropriate antibodies for 15 min on ice. Before analysis, cells were fixed in 1% paraformaldehyde. 10^4 cells/sample were analyzed on a FACScan® flow cytometer (Becton Dickinson and Co., San Jose, CA) using Lysys II software. The following mAbs were used: RA3-6B2 (B220, PE labeled; Pharmingen, San Diego, CA); 33-60 (anti-sIgM; FITC and biotin labeled; the hybridoma, 33-60 [11]); anti-sIgD (PE labeled; Southern Biotechnology Associates, Birmingham, AL); anti-CD23 (PE labeled; Pharmingen) M5-114 (anti-H-2^{bd}-FITC labeled). Biotinylated antibodies were visualized with streptavidin-PerCP (Becton Dickinson) or streptavidin-Quantum red conjugate (Sigma Chemical Co.).

BM Reconstitution Experiments. Donor bone marrow was panned on plates coated with anti-IgM, 33-60, for 1 h at 4°C to remove mature B cells. Nonadherent cells were harvested and resuspended in PBS at 5×10^6/ml. Recipients were irradiated with 900 rads and subsequently reconstituted with 2×10^6 bone marrow cells via tail vein injection. Mice were analyzed 9–11 wk after reconstitution by flow cytometric analysis of spleen and bone marrow.

Immunoprecipitation and Western Blotting. MB-1 immunoprecipitation was performed as described (12). A 1:50 dilution of rabbit antisera against mb-1 was used. Samples were boiled 5 min before separation of proteins on 10% polyacrylamide gels, followed by transfer to nitrocellulose by electroblotting for 2 h at 50 V. The membrane was blocked with wash buffer (20 mM Tris, 0.5 M NaCl, 0.1% Tween, pH 7.6) containing 3% gelatin for 1 h at room temperature. Antibody incubations were done in wash buffer containing 1% gelatin for 1 h at room temperature, followed by 5×5 min washes. Antiphosphotyrosine blots were performed using a 1/1,000 dilution of antiphosphotyrosine mAb 4G10 (Upstate Biotechnology Inc., Lake Placid, NY) followed by a 1/2,000 dilution of horseradish peroxidase (HRP)–coupled goat anti–mouse IgG (Sigma Chemical Co.). Bands were visualized by enhanced chemiluminescence detection system (NEN Dupont, Boston, MA). Blots were stripped by incubation for 15 min in 10 mM Tris-Cl pH 2.3; 150 mM NaCl, followed by washing five times in the same buffer at pH 8. For anti–phospholipase C (PLC)γ2 immunoprecipitation, lysis was performed in 0.5 ml of RIPA modified buffer (1% NP40, 0.25% sodium deoxycholate, 150 mM NaCl, 50 mM Tris, pH 7.5, containing protease inhibitors: 1 mM sodium orthovanadate, 1 mM PMSF, 10 μg/ml aprotinin/leupeptin) for 30 min on ice. Lysates were incubated with 15 μl rabbit antisera towards mouse PLCγ2 (Santa Cruz Biotechnology, Santa Cruz, CA), for 1 h at 4°C, followed by addition of protein A–Sepharose (Sigma Chemical Co.) for 1 h at 4°C. Immune complexes were washed three times with lysis buffer containing protease inhibitors followed by one wash in TN buffer (50 mM TrisCl, pH 7.5, 150 mM NaCl) containing protease inhibitors. Proteins were separated on 8% polyacrylamide gels. Antiphosphotyrosine blots were performed as described above. Anti-PLCγ2 blots were visualized by stripping and probing blots with anti-PLCγ2, followed by protein A–HRP.

Determination of $[Ca^{2+}]_i$. Cells at 5×10^6/ml in HBSS (pH 7.0) received the acetoxymethyl ester of indo-1 (Molecular Probes, Eugene, OR) to a final concentration of 1 μM. After 30 min at 37°C, an equal volume of HBSS containing 5% FCS (pH 7.4) was added to cells for an additional 30 min at 37°C. Cells were washed once in HBSS/FCS (pH 7.4), and resuspended in either HBSS/FCS (pH 7.4) or HBSS containing 0.5% BSA and 10 mM Hepes (pH 7.3). Before fluorescence measurements, aliquots of 5×10^5 cells were preincubated at 37°C for 1–2 min and were maintained at this temperature during measurement of the $[Ca^{2+}]_i$. Flow cytometric analysis of $[Ca^{2+}]_i$ was carried out using a FACStar® Plus (Becton Dickinson) using an ion laser (Innova Enterprise; Coherent, Santa Clara, CA) optimized for UV argon ions, set for 355-nm excitation at a power setting of 50 mW. Differential analysis of intracellular mobilization of Ca^{2+} vs extracellular influx was carried out by assessing the responsiveness of cells suspended in Ca^{2+}-free HBSS (buffered with 1.8 mM EGTA) followed by repletion of the external Ca^{2+} by addition of 4 mM $CaCl_2$. The response that occurs in Ca^{2+}-free medium reflects intracellular release.

Results and Discussion

Cell Surface Phenotype of CD45-deficient Cells. We have previously reported that normal numbers of B cells arise in CD45-deficient mice (9). We examined several cell surface antigens which characterize the progression of cells from the immature to the mature stage of B cell differentiation. The results revealed that high buoyant density splenic B cells obtained from CD45 −/− mice differed significantly from their normal littermate controls (Fig. 1). The majority of splenic B cells from normal mice were $B220^+$, δ^{hi}, μ^{lo}, and $CD23^+$. As expected, the B cells obtained from the CD45−/− mice failed to express the B220 antigen. However, we also found a significant decline in the frequency of the δ^{hi}, μ^{lo} population. In addition, there was a significant decline in B cells expressing CD23 in CD45 −/− mice. There was also a decline in the number of MHC class II^+ cells. Similar results were obtained for low buoyant density (1.079/1.066) B cells, whereas BM B cells were phenotypically normal (data not shown). These results raise the possibility that B cells in CD45−/− mice may have undergone a developmental arrest characterized by the transition from the immature to mature B cell stage (13).

T Cells Are Not Responsible for the Lack of Mature B Cells. CD45 −/− mice lack functional T cells (9). To determine if the B cell phenotype was due the lack of T cells we analyzed the B cell phenotype in irradiated recipients of bone marrow mixtures derived from CD45 −/− and normal mice. One of the most reliable markers for the CD45−/− B cell phenotype is the level of δ expression. In control studies in which the median δ expression was com-

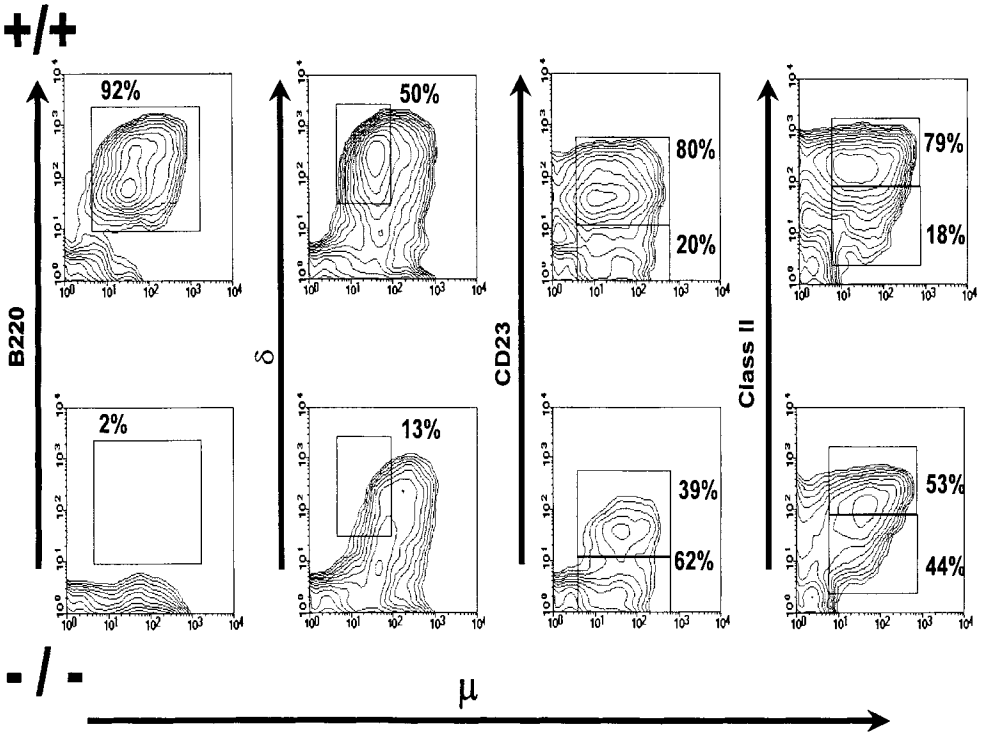

Figure 1. Flow cytometric analysis of high buoyant density (1.085/1.079) splenic B cells from $CD45^{+/+}$ and $CD45^{-/-}$ mice. The percentage of cells within the boxed regions are based on the total number of IgM^+ B cells. Mean fluorescent values determined for the following parameters were (+/+ vs −/−): μ (97 vs 135); δ (209 vs 78); CD23 (65 vs 23); and MHC class II (223 vs 120).

pared in age-matched normal and CD45−/− splenic B cells (five separate experiments, each with at least two spleens/group) the ratio of the median δ intensity of normal to CD45 −/− μ^+ B cells was 1.72 ± 0.6. A similar comparison of two normal irradiated/reconstituted mice, one reconstituted with +/+ bone marrow and one with −/− bone marrow resulted in a δ ratio of 1.6 (+/+ : −/−). This result indicates that irradiation/reconstitution protocol does not alter the levels of δ subsequently obtained. The median δ level was then determined in recipients (either +/+ or −/−) reconstituted (for 9–11 wk) with a 1:1 mixture of normal and CD45−/− B cell–depleted bone marrow. A comparison was made between μ^+B220^+ cells (derived from the normal bone marrow and representing ~40% of all B cells) and μ^+B220^- cells (derived from CD45−/− bone marrow and representing ~60% of all B cells). The ratio of the median δ intensity was found to be 1.70 ± 0.2. This result demonstrates that even in the presence of normal T cells and B cells, the CD45−/− B cells remain atypical.

BCR Cross-Linking Fails To Induce B Cell Proliferation. Density fractionation of splenic cells on Percoll gradients revealed that the CD45 −/− cells differed considerably from their normal counterparts. In four separate experiments, using a total of at least 10 individual spleens, we found a two- to fourfold reduction in the number of high

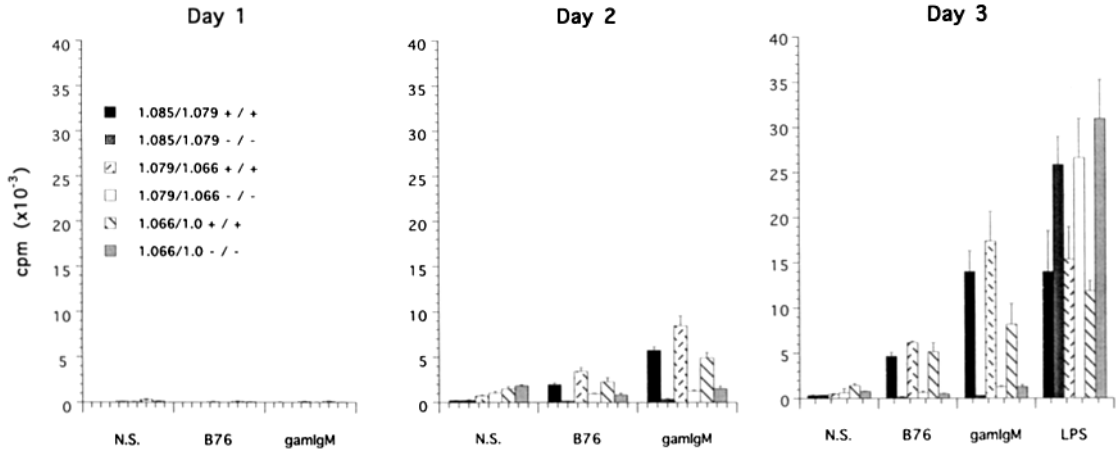

Figure 2. Igμ-induced proliferation of splenic B cells. Cells were cultured in either media alone (*N.S.*) or in the presence of 30 μg/ml of purified anti-μ, (*B76*), 30 μg/ml of polyclonal goat anti-mouse Igμ, or LPS (20 μg/ml). Incorporation of [^{3}H]thymidine was determined at 24, 48, or 72 h. All assays were performed in triplicate.

buoyant density (>1.079) B cells. In the same spleens, however, we found a two- to fivefold increase in the number of low buoyant density (1.079–1.066) B cells. Experiments using either monoclonal anti-μ or polyclonal reagents, revealed that neither the low nor the high buoyant density B cells incorporate [^{3}H]TdR upon cross-linking of Igμ (Fig. 2). It has been reported that immature cells fail to proliferate upon anti-Ig cross-linking (14). In this respect they are similar to the CD45 −/− B cells and might be used to support the conclusion that the B cells in CD45 −/− mice are developmentally arrested. Note, however, that immature cells express CD45 on their surfaces, albeit at lower levels (15). Thus the failure of immature cells to proliferate cannot be based on exactly the same parameters as the failure of CD45 −/− mice to proliferate.

Tyrosine Phosphorylation of mb-1 Occurs in Response to Ig-μ Cross-Linking. It has been reported that the Ig-associated protein, Igα (mb-1), is hyperphosphorylated on tyrosine in a B cell line deficient in CD45 expression (1). Furthermore, Igα was found to be hyperphosphorylated when CD45 was cross-linked on resting splenic B cells (2). These experiments suggested that CD45 may play a role in the maintenance of the basal level of tyrosine phosphorylation of Igα. In contrast to these reports, Igα was not hyperphosphorylated in B cells from CD45−/− mice (Fig. 3). Upon Igμ cross-linking, Igα is inducibly phosphorylated on tyrosine in both CD45+/+ and CD45−/− B cells. One explanation is that the population of B cells found in CD45 −/− mice may have undergone strong selection for alternative pathways which have replaced the need for CD45.

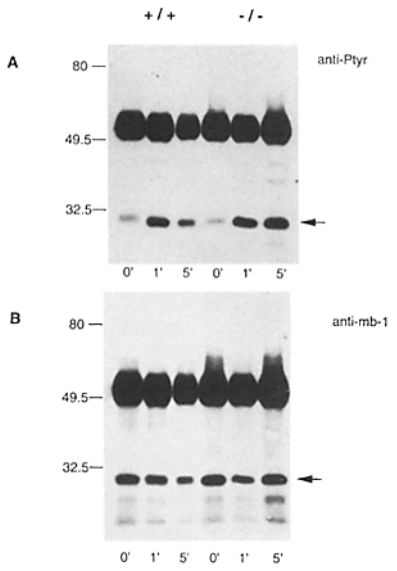

Figure 3. Induction of tyrosine phosphorylation of Ig-α. High buoyant density splenic B cells from CD45$^{+/+}$ and CD45$^{-/-}$ mice stimulated for various time periods at 37°C with 30 μg/ml of anti-μ, B76. Igα was immunoprecipitated with anti-Igα followed by separation of proteins on 10% SDS-PAGE. (*A*) Antiphosphotyrosine blot of immunoprecipitated Igα. (*B*) Blots were stripped and reprobed with anti-Igα followed by anti–rabbit HRP. The migration position of molecular weight markers are indicated on the left. The position of Igα is indicated by the arrow on the right.

According to this hypothesis, B cells from normal mice, having the luxury of using CD45 all their lives, may still depend on CD45 for the observed effects. Alternatively, the CD45-regulated Igα phosphorylation found by others may be unique to either the cell line used or to the method of manipulation of the splenic B cells in the sequestration studies.

Anti–Ig-Induced PLCγ2 Activation in CD45−/− B Cells. Another important signaling event that occurs upon Ig cross-linking is the tyrosine phosphorylation and activation of PLCγ2 (16–18). Results demonstrate that in response to Igμ cross-linking, a band of 140–150 kD, corresponding to PLCγ2, is inducibly phosphorylated in both CD45+/+ and CD45−/− B cells (Fig. 4). This suggested that upon Ig cross-linking, CD45 is not required for the induction of tyrosine phosphorylation of PLCγ2 in B cells.

Mobilization of Intracellular Calcium Is Normal but Influx from Extracellular Stores Is Abrogated in CD45-deficient B Cells. We examined Ca^{2+} mobilization in response to anti-Igμ cross-linking in high buoyant density splenic B cells. Treatment with anti-μ resulted in an increase in intracellular calcium concentration in both normal and CD45−/− B cells (Fig. 5 *A*). However, while the amplitude of the calcium mobilization in CD45−/− B cells was similar to that observed in normal B cells, Ca^{2+} levels returned to baseline values more rapidly in the CD45 −/− B cells. This result suggested that CD45 −/− cells may be unable to use extracellular stores of Ca^{2+}. This was directly tested by stimulating cells in the absence of extracellular Ca^{2+}. As shown in Fig. 5 *B* in the absence of extracellular Ca^{2+}, both normal and CD45 −/− B cells responded to anti-Igμ with a rapid and short-lived increase in $[Ca^{2+}]_i$. The addition of extracellular Ca^{2+} to normal cells (*arrow b*) resulted in a second wave of increased $[Ca^{2+}]_i$ reflecting the use of extracellular Ca^{2+}. In contrast, the addition of extracellular Ca^{2+} does not affect $[Ca^{2+}]_i$ levels in CD45−/− B cells. Previous reports failed to reach a consensus regarding the role of CD45 in mobilization of Ca^{2+}. The J558Lμm3 plasmacy-

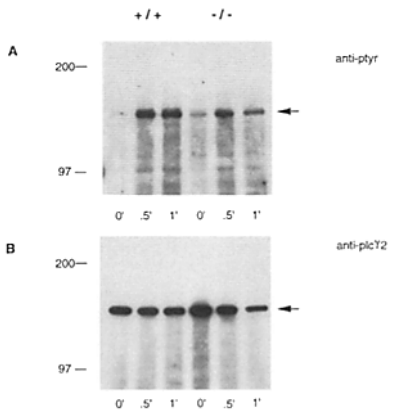

Figure 4. Induction of tyrosine phosphorylation of PLCg2. Whole splenic B cells were stimulated using goat anti–mouse IgM. (*A*) Antiphosphotyrosine blot of immunoprecipitated PLCγ2. (*B*) Blots were stripped and reprobed with anti-PLCγ2 followed by protein A–HRP. The arrow on the right represents the position of PLCγ2.

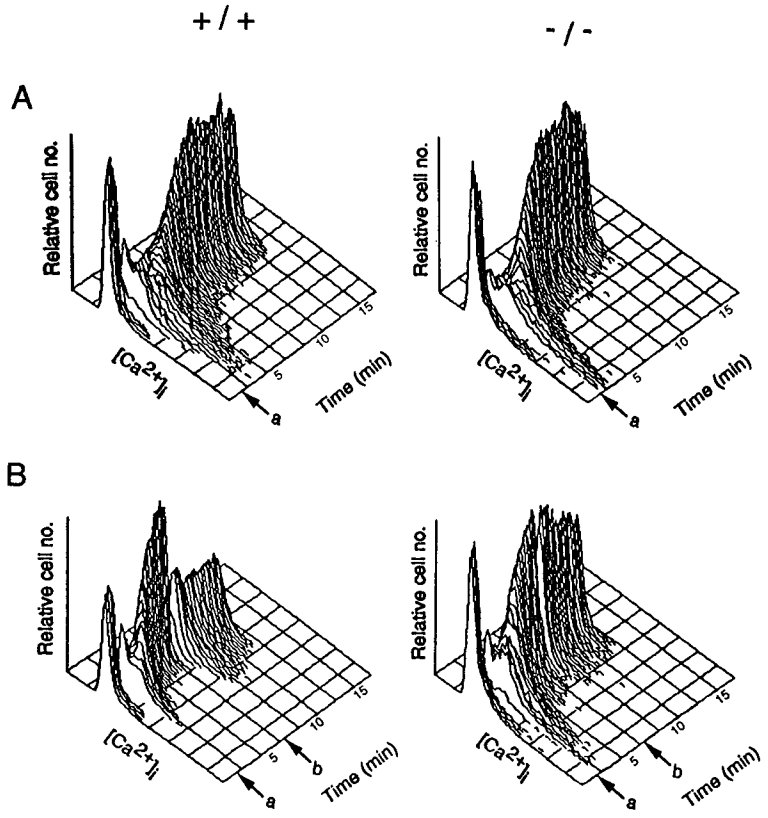

Figure 5. Calcium mobilization in response to Igμ cross-linking. High buoyant density splenic B cells were loaded with indo-1, and stimulated with anti-μ, B76 (40 μg/ml) (*A, arrow a*), or B76 (40 μg/ml) + EGTA 1.8 mM (*B, arrow a*) followed 5 min later by addition of 4 mM $CaCl_2$ (*B, arrow b*). Intracellular calcium increases were measured as the ratio of fluorescence of calcium-bound indo-1/calcium-free indo-1 (405:530 nm) by flow cytometry.

toma requires CD45 for Ca^{2+} mobilization (1). In contrast, the immature cell line WEHI 231, has a slightly delayed but strong Ca^{2+} mobilization response (8). Human tonsillar B cells have reduced anti–Ig-mediated Ca^{2+} mobilization after pretreatment with anti-CD45 antibodies (6). These reports provided no indication that intracellular and extracellular sources of Ca^{2+} may be subject to different regulatory mechanisms. Our observation that the mobilization of intracellular stores of Ca^{2+} is CD45 independent, but that subsequent influx of Ca^{2+} from extracellular sources is CD45 dependent, reveals a previously unrecognized regulatory event in B cell activation. The absence of this event in CD45 −/− mice may arrest B cells at a stage of development which normally would be characterized by the transition from the immature to mature splenic B cell (13). This raises the possibility that such events may be regulated by the nature of external stimuli received by cells undergoing this transition.

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