

Abnormal Transcription Factor Induction through the Surface Immunoglobulin M Receptor of B-1 Lymphocytes

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Summary

Populations of murine peritoneal B-1 and splenic B-2 cells, highly purified by negative selection techniques, were used to demonstrate that B-1 cells completely fail to enter cell cycle in response to surface immunoglobulin M (sIgM) crosslinking without any decrease in cell number or viability. This failure of B-1 cell responsiveness appears to represent a specific defect in sIgM-derived signaling inasmuch as stimulation to enter S phase occurs normally in response to activated and fixed T cells, and to lipopolysaccharide (LPS). The level at which sIgM signaling fails was determined by evaluating the nuclear expression of the transcription factor complex, NF- κ B, whose sIgM-mediated induction in B-2 cells is dependent on protein kinase C (PKC) activation but is independent of protein synthesis. There was no induction of nuclear NF- κ B in B-1 cells stimulated by sIgM crosslinking, although NF- κ B was stimulated by phorbol myristate acetate and by LPS. In contrast, NF- κ B was induced in B-2 cells by all three stimuli. Thus, in B-1 cells, the sIgM-mediated induction of a transcription factor that is substantially stimulated by anti-IgM in B-2 cells is blocked. However, all sIgM-derived signaling in B-1 cells was not impaired inasmuch as anti-IgM increased I-A antigen expression. These results strongly suggest that sIgM receptor-mediated signaling in B-1 cells is interrupted early in the signal transduction pathway, at a point proximal to the activation of PKC. These results further demonstrate that transcription factor induction can be used to analyze the level at which receptor-mediated signaling is blocked.

Receptor-mediated induction of cell cycle progression is accompanied by alterations in gene expression that appear to be brought about by *trans*-acting transcription factor complexes. Nuclear expression of one such complex, NF- κ B, is stimulated early (within the first 2 h) after surface IgM (sIgM) receptor crosslinking in primary, conventional B cells (1, 2), and this induction appears to be protein kinase C (PKC) dependent (1, and our unpublished observations). As shown by others, nuclear expression of NF- κ B likely results from the PKC-mediated modification of an inhibitor (I κ B) associated with NF- κ B, the NF- κ B/I κ B complex being constitutively present in the cytosol (3); thus, this factor represents a fairly direct path from receptor-mediated kinase activation to alteration in gene expression that is not dependent on protein synthesis (1).

B-1 cells (formerly termed Ly-1⁺ or CD5⁺ B cells) differ in many respects from conventional B (B-2) cells and may represent a unique, early appearing lymphoid lineage (reviewed in reference 4). It has been suggested that, unlike B-2 cells, B-1 cells fail to progress in cell cycle in response to sIgM crosslinking (5–8). However, these reports were based on studies using peritoneal B lymphocytes that were not depleted of

“contaminating” conventional B cells or B-1 cells that were purified by positive selection for Ly-1 expression; the latter may have the potential to alter the normal responsiveness of these cells to other receptor-mediated signals. In the present work we have used peritoneal B-1 lymphocytes purified by negative selection techniques to define the specificity and the extent of the sIgM-mediated signaling defect in these cells. In particular, the induction of NF- κ B was assessed to determine whether the defect in sIgM signaling in B-1 cells lies proximally or distally to the receptor-mediated activation of PKC.

Materials and Methods

Animals. Male BALB/cByJ mice at 8–14 wk of age were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were rested at least 1 wk before experimentation.

B Cell Purification. B-1 lymphocytes were prepared using a modification of previously described methods (9). Peritoneal wash-out cells were depleted of T cells with anti-Thy-1.2 plus rabbit complement, depleted of macrophages by overnight adherence to plastic, and depleted of residual macrophages and other nonlymphoid cells by sedimentation on discontinuous Percoll gradients (with densi-

ties of 1.10, 1.09, 1.08, and 1.069 g/ml) followed by harvesting of cells at the 1.069/1.08 interface. Conventional B cells were removed by incubation with biotin-coupled rat anti-mouse (RaM) CD23 and rat anti-mouse IgD⁺ (Pharmingen, San Diego, CA) in the presence of rat gamma globulin (Jackson ImmunoResearch, West Grove, PA), followed by streptavidin magnetic particles (Advanced Magnetics, Cambridge, MA), and removal on a magnetic concentrator (Dynal, Great Neck, NY). B-2 cells were similarly purified from BALB/cByJ splenocytes, except that red blood cells were removed by hypotonic lysis using Gey's solution, the 1.069/1.08 and the 1.08/1.09 Percoll interfaces were combined, and contaminating B-1 cells were removed with biotin-coupled rat anti-mouse Ly-1 (Pharmingen) plus streptavidin magnetic particles.

Flow Cytometry. Cells were labeled with FITC-conjugated F(ab')₂ goat anti-mouse (GaM)IgM (Jackson ImmunoResearch) or PE-conjugated F(ab')₂ GaMIgM (Jackson ImmunoResearch), FITC-F(ab')₂ GaMIgD⁺ (Pharmingen), and either biotin-RaM-CD23 (Pharmingen) or biotin-RaM-CD5 (Pharmingen) plus biotin-RaM-Mac-1 (kindly provided by Dr. Henry H. Wortis, Tufts University School of Medicine, Boston, MA), followed by streptavidin(SA)-PE (Pharmingen), as previously described (10). Non-viable cells were eliminated by gating on the basis of propidium iodide exclusion. Quadrant analysis for specifically stained cell populations was carried out on the basis of <2% nonspecific staining with biotin-RaM-CD8 (Pharmingen) plus SA-PE as a negative antibody control.

Measurement of Stimulatory Responses. Proliferative assays were carried out as previously described (11) using 10⁵ B cells/culture. Murine T cells (CDC35 Th2 clone) were kindly provided by Dr. David C. Parker (University of Massachusetts Medical School, Worcester, MA). T cells were activated by solid phase anti-CD3 antibody and fixed with formaldehyde as described (12).

Electrophoretic Mobility Shift Assays (EMSA). Nuclear κ B-binding activity was detected by the electrophoretic gel mobility shift assay as previously described (1), except that nucleoprotein complexes were separated on 4% native polyacrylamide gels. κ B-binding activity was quantitated by densitometric scanning of autoradiograms (Molecular Dynamics, Sunnyvale, CA).

Reagents. PMA and LPS (*Escherichia coli*, serotype 0127:B8) were obtained from Sigma Chemical Co. (St. Louis, MO). Affinity purified F(ab')₂ GaMIgM was obtained from Jackson ImmunoResearch. Percoll was obtained from Pharmacia Fine Chemicals (Piscataway, NJ).

Results and Discussion

Purification of B-1 and B-2 Cell Populations. Purified B-1 cells were derived from peritoneal B lymphocytes by depletion of "contaminating" conventional B (B-2) cells using negative selection techniques based on the marked variation in expression of CD23 and sIgD between these populations, as described in Materials and Methods. Purified B-2 cells were obtained from splenic B lymphocytes by depletion of Ly-1⁺ cells. The purity of these two B cell populations was assessed by histologic examination of cytocentrifuge preparations and by flow cytometric analysis of surface antigen expression. Purified B-1 cells were found to be >99% lymphocytes and <0.5% macrophages, neutrophils, and basophils. These cells were >99% sIgM⁺ and 94% positive after staining with anti-Ly-1 plus anti-Mac-1 (Fig. 1 A). Another 4% consisted of cells that were Ly-1⁻ and Mac-1⁻ but expressed levels of

sIgM >99% of conventional B cells. Thus, at least 94% of the B-1 population represents either B-1a or B-1b ("sister") cells, and (on the basis of high-density sIgM expression) the B-1 population may be 98% pure. Purified B-2 cells were found to be >99% sIgM⁺ and predominantly CD23 expressing (87%) and sIgD^{hi}.

Characterization of B-1 Stimulatory Responses. The responses of these purified populations were assessed by measuring the incorporation of [³H]thymidine after treatment with various mitogens. B-1 cells completely failed to respond to several doses of anti-IgM that were fully stimulatory for B-2 cells (Fig. 1 B), while viability was not affected (Table 1). B-1 cells were not inert to stimulation, however, as they responded vigorously to PMA, which, as shown previously, is a sufficient mitogen for B-1, but not B-2, cells (10, 11). B-1 and B-2 cells responded well to LPS stimulation, and further, the two B cell populations responded comparably to stimulation by activated and fixed T cells (Fig. 1 C), which has been shown to involve a specific ligand/receptor (p39/CD40) interaction (12, 13). These results verify the existence of a defect in sIgM-mediated signaling using negatively selected B-1 cells that are virtually devoid of B-2 cells and are not subject to the potentially confounding influence of CD5 ligation. Moreover, the lack of S phase entry in response to anti-IgM is shown here to represent a block in B-1 cell signaling that is specific for and restricted to the sIgM receptor.

Abnormal Induction of Nuclear NF- κ B Expression in B-1 Lymphocytes. In peritoneal B cells sIgM crosslinking leads to a seemingly contradictory increase in intracellular Ca²⁺ (14, 15) without an apparent increase in inositol phosphate generation (our unpublished observations). To clarify the level at which sIgM signaling is blocked, the sIgM-mediated induction of nuclear NF- κ B was evaluated. We reasoned that if transcription factor induction is normal (conventional B cell-like), then the defect in sIgM signaling is likely to be located downstream, perhaps within the genome (e.g., due to methylation of a gene that is normally expressed after antigen receptor crosslinking); whereas if transcription factor induction is abnormal, then the defect likely lies between sIgM and PKC-mediated phosphorylation.

The EMSA was used to detect NF- κ B with a κ B-containing oligonucleotide (1). There was little or no induction of nuclear κ B-binding activity in B-1 cells after crosslinking of sIgM (Figs. 2 and 3). However, κ B-binding activity was effectively induced in B-1 cells by PMA and by LPS. In direct and marked contrast, sIgM ligation mediated rapid induction of κ B-binding activity in B-2 cells, with a peak at 2–4 h, as did PMA and LPS. Although there was less PMA-induced κ B-binding activity in B-1 as opposed to B-2 cells in some experiments, induction over respective baseline values was similar in the two B cell populations, owing to the lower constitutive expression of NF- κ B in B-1 cells when similar quantities of nuclear-extracted protein were evaluated. To control for variation in the absolute level of PMA-induced κ B-binding activity between the two B cell populations, κ B-binding activity observed after treatment with anti-IgM was expressed as a proportion of that stimulated by PMA. In B-1

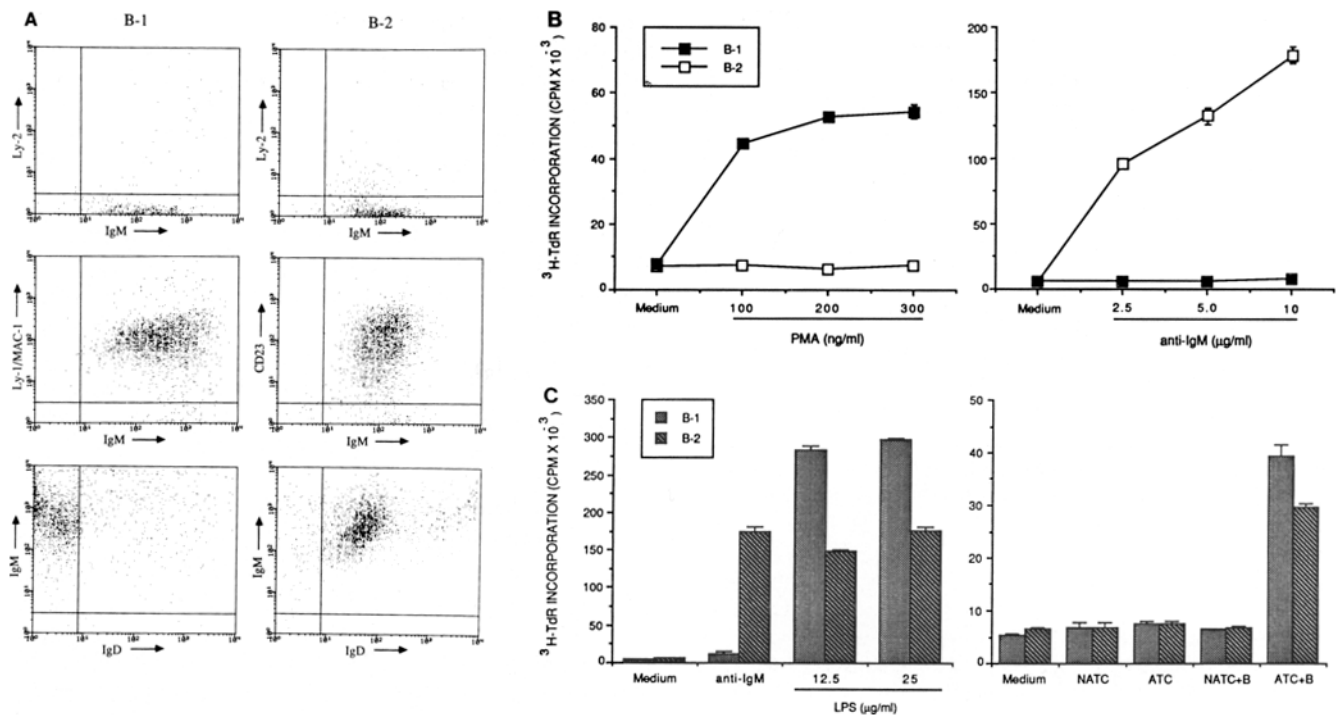


Figure 1. (A) Phenotypic analysis of purified B-1 and B-2 cells. Purified B-1 and B-2 cells were labeled with fluorescent antibodies as indicated, and analyzed by flow cytometry as described in Materials and Methods. (B) S phase entry of B-1 and B-2 cells. Purified B-1 and B-2 cells were stimulated with PMA (100–300 ng/ml) for 24 h or F(ab')₂ fragments of GaMIgM (2.5–10 μg/ml) for 48 h. (C) S phase entry of B-1 and B-2 cells. Purified B-1 and B-2 cells were stimulated with F(ab')₂ GaMIgM (10 μg/ml), LPS (12.5 and 25 μg/ml), and either fixed nonactivated T cells (NATC) or fixed activated T cells (ATC) at a 1:1 (T/B) ratio, for 48 h. In B and C, [³H]TdR incorporation during the last 6 h of culture was measured. These data are presented as the mean ± SEM of quadruplicate cultures reported as cpm (×10⁻³). All data presented are representative of at least three separate experiments.

cells, κB-binding activity in the presence of anti-IgM (no induction) amounted to only one-fourth to one-fifth of that stimulated by PMA at 2 h (0.26 ± 0.06 , $n = 5$) and at 4 h (0.20 ± 0.03 , $n = 4$), while in B-2 cells, anti-IgM produced levels of κB-binding activity that were equal to those stimulated by PMA at 2 h (1.06 ± 0.09 , $n = 5$) and at 4 h (1.03 ± 0.08 , $n = 4$), as determined by densitometry.

To rule out the possibility that the peak of induced κB-binding activity was shifted in B-1 cells, additional experiments were carried out that included shorter and longer exposures to anti-IgM. In addition to the time points shown (Fig. 3), there was no sIgM-mediated induction of κB-binding activity in B-1 cells after 0.5 h of stimulation (data not shown).

Constitutive and inducible κB-binding activity in B-1 and B-2 cells was appropriately competed by wild-type, but not by mutated, κB-containing oligonucleotide (data not shown). This, along with the electrophoretic mobility of the nucleoprotein complexes, suggests that the activity detected by EMSA represents NF-κB.

These results indicate that NF-κB is capable of nuclear translocation and induction in B-1 cells after direct PKC activation (by PMA) or through a non-PKC-dependent pathway (by LPS), but not as a result of sIgM crosslinking. Since in B-2 cells sIgM-mediated induction of nuclear NF-κB is PKC

dependent, this strongly implies that sIgM-derived signals fail to adequately activate PKC in B-1 cells.

Induction of MHC Class II (I-A) Antigen Expression by B-1 Lymphocytes. To determine whether all forms of sIgM-mediated signaling are blocked in B-1 cells, additional studies were conducted to evaluate the expression of class II MHC (I-A) antigens by flow cytometry. In contrast to a previous report on unseparated peritoneal B cells, purified B-1 cells were found to express higher basal levels of surface I-A than B-2 cells (16). After stimulation with anti-IgM for 24 h, the level of B-1 I-A expression increased (Table 1). The increase in I-A expression was similar to that induced by IL-4 and by PMA in B-1 cells, but in three separate experiments was less than the increase induced in B-2 cells by these same reagents. This effect however, was not specific for sIgM-derived signals, and thus likely reflects intrinsic differences in induction of I-A antigen expression between the two B cell populations.

Further evidence that the sIgM receptor of B-1 cells is not inert lies in our recent observation that anti-IgM stimulates similar patterns of protein tyrosine phosphorylation as that seen in B-2 cells (our unpublished observations), and in previous reports that receptor ligation leads to changes in intracellular Ca²⁺ (14, 15) and to antigen presentation (17). Thus, the failure of intracellular signaling is not only a de-

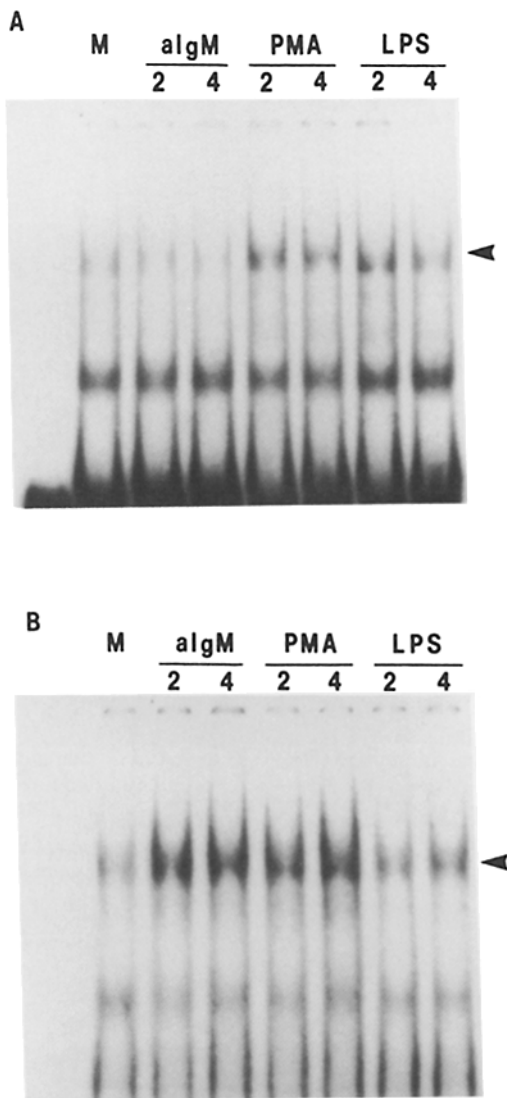


Figure 2. Induction of nuclear κ B-binding activity in B-1 and B-2 lymphocytes. Purified B-1 (A) and B-2 (B) cells were cultured (4×10^6 /ml) in the presence of medium alone (M) or medium containing either F(ab')₂ GaMIgM (aIgM; 10 μ g/ml), PMA (200 ng/ml), or LPS (25 μ g/ml) for 2 and 4 h. Cells were washed and nuclear extracts prepared as previously described (1). Extracts were tested by EMSA using a κ B-containing oligonucleotide probe. The data presented are representative of four separate experiments, including the data shown in Fig. 3.

Table 1. Induction of I-A Antigen Expression in B-1 and B-2 Cells

Exp.	Treatment	Cell viability	Median fluorescence intensity (MFI)	Δ MFI
		%		
B-1	Medium	87	661	-
	PMA	94	717	56
	IL-4	86	721	60
	Anti-IgM	89	705	44
	Medium	90	686	-
B-2	Anti-IgM	84	751	67
	Medium	73	564	-
	PMA	81	657	93
	IL-4	77	655	91
	Anti-IgM	54	679	115
	Medium	73	598	-
	Anti-IgM	76	721	123

B-1 and B-2 lymphocytes were cultured at 2×10^6 cells/ml in medium alone or medium containing PMA (50 ng/ml), IL-4 (50 U/ml), or F(ab')₂ GaMIgM (10 μ g/ml) for 24 h. Cells were harvested and labeled with anti-I-A^d and analyzed by flow cytometry as described in Materials and Methods. Data are summarized and presented as the difference (Δ) in median fluorescence intensity (MFI) between stimulated and unstimulated cells. The viability of recovered cells was assessed by trypan blue dye exclusion. Two representative studies are presented.

fect specific to B-1 cell sIgM, it appears to be specific for sIgM-derived signals that lead to proliferation.

Cell viability was assessed in connection with phenotypic evaluation by determining the trypan blue dye exclusion of recovered cells. As shown in Table 1, the viability of B-1 cells was not affected by anti-IgM treatment, and viable cell recovery of anti-IgM-treated B-1 cells was, in fact, greater than that observed for B-2 cells. Thus, there is no evidence that sIgM crosslinking by F(ab')₂ fragments of anti-IgM antibodies in vitro is deleterious for B-1 cells. Although this contrasts with

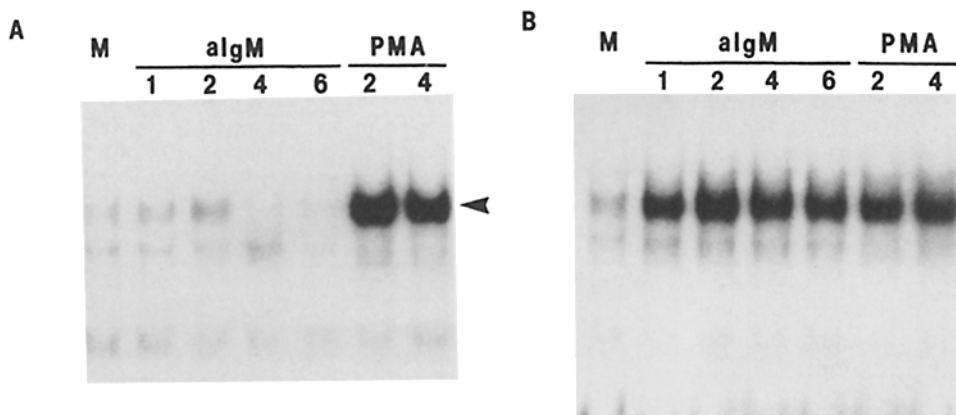


Figure 3. Induction of sIgM-mediated nuclear κ B-binding activity in B-1 and B-2 lymphocytes. Purified B-1 (A) and B-2 (B) cells were cultured in the presence of medium alone (M) or medium containing either F(ab')₂ GaMIgM (aIgM; 10 μ g/ml), for 1, 2, 4, and 6 h, or PMA (200 ng/ml) for 2 and 4 h. Nuclear extracts were prepared and analyzed as described in Fig. 2.

the report that antigen-mediated sIgM interaction leads to apoptosis of B-1 cells (18), that report is inconsistent with the observation that B-1 cells are not tolerizable as a result of sIgM crosslinking (15), and it may be that the apoptosis observed by Murakami et al. (18) involves additional cytokine-mediated influences or FcR-mediated signaling resulting from antigen-bound autoantibody.

The present work demonstrates that a specific defect in sIgM signaling for proliferation exists in B-1 cells and that this defect is associated with an inability to properly stimulate nuclear translocation of a constitutively expressed *trans*-acting transcription factor whose induction is normally dependent on PKC. This strongly suggests that the block in B-1 sIgM-mediated signaling occurs at a level proximal to the activation of PKC, a conclusion supported by the observation that direct stimulation of PKC leads to strong induction of NF- κ B in B-1 cells.

At this point it cannot be said that the failure of NF- κ B induction is responsible for the lack of B-1 cell cycle progression; however, the analysis of transcription factor induction described herein provided the means to localize the level of defective sIgM signaling by focusing on a physiological outcome of receptor-mediated kinase activation. This approach may be generally applicable to the dissection of other defects in receptor-mediated immune cell responsiveness.

Despite the common occurrence of autoreactive antigen receptors, B-1 cells are not unduly expanded in vivo under normal conditions. Thus, the block in sIgM-mediated signaling as characterized in this report may be a physiological mechanism that prevents untoward stimulation and clonal expansion of B-1 cells by autologous antigen in vivo.

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