Induction of B cell apoptosis by co-cross-linking CD23 and slg involves aberrant regulation of c-myc and is inhibited by bcl-2

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Abstract

A novel system to study the effects of co-cross-linking CD23/FcɛRII and slg on murine B lymphocytes utilizes a highly multivalent form of anti-Ig prepared by covalently linking anti-Ig antibodies to a DNP-dextran backbone. CD23–slg co-cross-linking is accomplished by the addition of DNP-specific monoclonal IgE. Previous studies demonstrated that co-cross-linking CD23 and slg significantly inhibited mouse B cell proliferation, especially at high doses of the multivalent anti-Ig. Interestingly, examination of early activation signals reveals no difference in B cells subjected to co-cross-linking conditions as compared to B cells activated with anti-Ig alone. Total cellular protein tyrosine phosphorylation levels are unchanged by co-cross-linking. Analysis of B cell mRNA reveals that co-cross-linking the receptors does not alter the expression levels of ornithine decarboxylase 8 h after stimulation as compared to the controls. In contrast, levels of the proto-oncogene c-myc were significantly elevated 1 h after inducing B cell activation under co-cross-linking conditions. However, it remains unclear whether this aberrant c-myc regulation plays any role in inducing apoptosis. In addition, on day 3 after stimulation, the co-cross-linking of CD23 and slg resulted in the formation of apoptotic B cells, determined by both photomicroscopy of the B cell cultures and FACS analysis of B cell nuclei. B cells obtained from bcl-2 transgenic mice proliferated as well as controls, and failed to undergo apoptosis when CD23 and slg were co-cross-linked on their surface. These studies indicate that co-cross-linking of CD23 with B cell slg inhibits B cell proliferation by a mechanism that is distinct from that seen by co-cross-linking of the FcγRII and slg. In addition, these results suggest a means by which antigen-specific IgE can down-regulate additional B cell activation and IgE synthesis.

Introduction

Stimulation of B cells by cross-linking their slg receptors has proven to be a useful model to study B cell activation events. A very efficient way to activate B cells via slg is to use a highly multivalent anti-slg system such as anti-IgD-dextran, first described by Brunswick et al. (1). We took advantage of this system in order to investigate the effects of co-cross-linking of the low-affinity receptor for IgE (FcɛRII/CD23) and slg (2). In this model, monoclonal anti-IgD was coupled to DNP-dextran (HO[5/1-{DNP-dextran}]), and co-cross-linking of slg and CD23 resulted upon addition of anti-DNP IgE to B cells activated by the anti-IgD–DNP-dextran. B cell activation was found to be strongly inhibited by CD23-slg co-cross-linking, however, in contrast to the FcγRII co-cross-linking system (3,4), IL-4 did not reverse the inhibition (2).

In the studies reported here, we further investigate the cellular mechanisms that mediate the negative effect of co-
cross-linking CD23 and slg. Tyrosine phosphorylation is now believed to be one of the earliest events to occur subsequent to B cell activation by slg. No change was seen in this early activation signal under CD23–slg co-cross-linking conditions. In addition, examination of a later activation event, i.e. the expression of the enzyme ornithine decarboxylase (ODC), was also unaffected. However, the proto-oncogene c-myc expression of the enzyme ornithine decarboxylase (ODC), was aberrantly up-regulated, increasing to a level that was 2–3-fold above levels observed when slg alone is cross-linked. Overexpression and/or aberrant regulation of c-myc has frequently been implicated in the mechanism of programmed cell death or apoptosis. Apoptosis is an active process through which cells ‘commit suicide’ and provides a very effective means of controlling biological systems in a multicellular organism. For instance, programmed cell death is important in the elimination of cells during embryonic development and in the deletion of T cells during thymic selection (5,6). Fas is thought to play an important role in this apoptosis and may play a role in B cell apoptosis models as well (7). Evolutionarily, the information necessary to activate cell death is encoded in all cells. While triggering mechanisms may differ, cells that undergo apoptosis experience similar alterations in morphology. The distinguishing characteristics of apoptosis include condensation of cytoplasm, loss of plasma membrane microvilli and degradation of nuclear chromatin (6,8). By several different criteria, we demonstrate here that increased cell death via apoptosis occurs in B cells subjected to CD23–slg co-cross-linking. Finally, we demonstrate that induction of apoptosis by co-cross-linking CD23 and slg is completely blocked in mice overexpressing the oncogene bcl-2.

Methods

Antibodies

H63/1 and AF3.33 are allotypic anti-IgDα (9) and IgDβ (10) mAb respectively. Coupling of the anti-IgD to DNP–dextran was performed as described (2). Rat IgE anti-DNP (11) was isolated from ascites (12). Rabbit anti-IgM F(ab′)2 was prepared in-house as described previously (2). Mouse anti-rat κ-light chain (Mar 18.5), anti-Thy-1.1 (clone TIB99), anti-CD8 and anti-CD5 were all gifts of Dr William Paul, and have been described previously (13).

Mice

Female BALB/c and C57Bl/6 mice were purchased from the National Cancer Institute (Frederick, MD), and were used between 6 and 8 weeks of age. Mice transgenic for bcl-2 on a C57Bl/6 genetic background (14,15) were obtained from the WEHI (Melbourne) and were maintained under pathogen-free conditions at Immunex. Mice transgenic for p53 (C57Bl/6 background) were obtained from Taconic (Germantown, NY). All transgenic mice were used between 6 and 8 weeks of age, prior to the development of any lymphomas.

Cell preparation and cell culture

B lymphocytes from normal and transgenic mice were purified from naive spleen cell suspensions using a modification of the negative selection procedure described by Gold et al. (16) and fully described elsewhere (17). Briefly, spleens were passed through sterile wire mesh screens and the cells were resuspended in an antibody cocktail containing anti-Thy-1.2, anti-CD5 and anti-CD8 mAb. T cell depletion was accomplished through the addition of guinea pig complement (Life Technologies, Gaithersburg, MD) and MAR 18.5, a rat anti-κ light chain mAb (18) added to enhance complement activation. B cells were separated by density using sedimentation on discontinuous Percoll gradients containing 50, 60, 66 and 70% Percoll (Sigma, St Louis, MO). Resting B cells were recovered from the 66/70% interface. The resulting population was 85–95% B220+ as determined by flow cytometry. T cell contamination was <2%. High density B cells were utilized in all studies described herein. Unless otherwise noted, B cell assays were performed in RPMI 1640 containing 10% fetal bovine serum (Hyclone, Logan, UT), 100 U/ml penicillin and streptomycin (Life Technologies), 10 mM sodium pyruvate (Sigma), 1 mM oxalacetic acid (Sigma), 5 × 105 M β2-mercapto-ethanol (Sigma), 2 mM glutamine (Life Technologies) and 50 μg/ml gentamycin (Sigma).

Isolation of B cell proteins for analysis of tyrosine phosphorylation

B cells isolated as described above were seeded in Costar (Cambridge, MA) six-well plates at 1 × 106 cells/ml in protein-free HBSS in a final volume of 5 ml. The cells were incubated overnight at 37°C in the presence of 500 U/ml IL-4, a generous gift from Dr William Paul (NIH, Bethesda, MD), and appropriate cultures were pretreated with 10 μg/ml rat IgE anti-DNP for 2 h prior to addition of the appropriate stimulants. Once indicated stimulants were added, reactions were allowed to proceed for ~10 min at 37°C. Reactions were stopped by addition of the cells to a tube containing 10 ml of ice-cold PBS containing Na3VO4. Cells were resuspended in 0.2 ml lysis buffer (20 mM Tris–HCl, pH 8, 137 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, 20 μM leupeptin and 0.15 U/ml aprotinin, all purchased from Sigma) and incubated 10 min on ice; after removal of nuclei by centrifugation, protein concentrations of Triton X-100 soluble fractions were determined using the microtiter well Bradford Assay (Bio-Rad, Melville, NY).

Western analysis of B cell proteins

From each assay condition, 50 μg of Triton X-100 soluble proteins was separated by SDS–PAGE using an 8–18% acrylamide gradient gel. Separated proteins were then transferred to a 0.45 μm nitrocellulose membrane (Schleicher & Schuell, Keene, NH). Non-specific sites on the nitrocellulose were blocked using TBST (50 mM Tris–HCl, pH 7.6, 0.15 M NaCl and 0.05% Tween-20) supplemented with 4% horse serum (HS) from Atlanta Biologicals (Nocross, GA). The blot was incubated at room temperature with the primary antibody, monoclonal anti-phosphotyrosine (UBI, Lake Placid, NY) diluted 1:1000 in TBST/4% HS. After washing to remove unbound primary antibody, detection of bound anti-phosphotyrosine was accomplished using a 1:2000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG (heavy chain specific; Southern Biotechnology Associates, Birmingham, AL) resuspended in TBST/4% HS. After washing, the blots were developed by addition of diaminozenbri-
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Measurement of B cell apoptosis

Several methods were employed to assess apoptosis. Cultured cells were examined directly by microscopy after the indicated days of culture. Contents of a single culture well were collected by cytocentrifugation and the cells were stained with May–Grunwald Giemsa. Alternatively, cytocentrifuged cells were fixed with 4% buffered formalin for 10 min then stained using an ApopTag kit (Oncor, Gaithersburg, MD) according to the manufacturer’s directions. This procedure involves end-labeling the DNA with digoxigenin–UTP in the presence of TD T and staining the permeabilized cells with FITC–anti-digoxigenin.

Apoptosis in B lymphocytes was also assessed using a flow cytometric procedure. According to a procedure originally reported by Nicoliceti et al. (19) and modified by Ashman (University of Iowa) (20), purified splenic B cells were cultured in 24-well plates in a final volume of 1.5 ml. Indicated stimulants were added and the cells were harvested after 3, 4 or 5 days in culture. Cell pellets from each culture condition were resuspended in 1.5 ml of a hypotonic fluorochrome solution containing 50 μg/ml propidium iodide (Sigma), 0.1% sodium citrate (Sigma) and 0.1% Triton X-100. Cell suspensions were then incubated at 4°C to arrest further DNA fragmentation and to remove plasma membranes. Resulting nuclei were analyzed on a Becton Dickinson (San Jose, CA) FACSscan set on low flow speed. The FACSscan was calibrated using CaliBRITE beads from Becton Dickinson and analysis was done with FACSscan Research Software. Nuclei from cells undergoing apoptosis characteristically have hypodiploid DNA which induce an increase in side scatter and a decrease in forward light scatter. In contrast, nuclei from normal or actively proliferating cells generate a profile of increased forward light scatter and decreased side scatter.

Assessment of apoptosis by multiparameter flow cytometry

B cells purified from the spleens of C57BL/6 and bcl-2 transgenic mice as described were cultured for 48–72 h in Costar 24-well plates at 5 × 10⁵ cells/ml in the presence of the indicated stimulus and cytokines. Assessment of apoptosis was accomplished using a multiparameter flow cytometric technique previously described by Dive et al. (21). Briefly, B cells were collected by centrifugation and then resuspended in 0.5 ml FACS buffer (PBS, 5% FCS, 1% formaldehyde). Prior to analysis, Hoechst 33342 (Calbiochem, La Jolla, CA) and propidium iodide were added to single-cell suspensions to final concentrations of 10 μM and 20 μg/ml respectively.

Samples were incubated at 37°C for 7 min and then analyzed on a Coulter Epics Elite cytometer equipped with both argon (488 nm emission) and helium–cadmium (325 nm emission) lasers. Forward light scatter and log red fluorescence (Hoechst 33342; 525 nm) were measured at a rate of 700–800 cells/s. Analysis was with Coulter Software. Cell debris and doublets were excluded from analysis by light scatter; dead and necrotic cells by propidium iodide gating. Data are presented as two-dimensional contour plots of forward light scatter versus log blue fluorescence.

RNA isolation

B lymphocytes were cultured at 10⁶ cells/ml (2–4 × 10⁷ cells total per assay condition) in Costar six-well plates in the presence of 500 U/ml IL-4 for 24 h. To the appropriate cultures, 10 μg/ml rat IgE anti-DNP was added 2 h prior to addition of indicated stimulants (19). The reactions were stopped at the indicated time points by addition of ice-cold PBS. Cells were collected and washed once with PBS prior to isolation of total cellular RNA by the guanidine thiocyanate method with the cesium chloride modification. Briefly, cells from each culture condition were washed once with PBS then resuspended in 2.5 ml of a 4 M guanidine thiocyanate (Sigma) solution containing 10 mM Tris, pH 7.4, and 7% 2-mercaptoethanol. In order to disrupt cellular proteins and sheer DNA, 270 μl of 20% lauryl sarcosine (Sigma) was added and each suspension was vigorously passed through a 22-gauge needle repeatedly until no longer viscous. Each sample was then layered onto 500 μl 5.7 M cesium chloride (Sigma) in 10 mM EDTA, pH 7.4, in Beckman Konical tubes (13 × 51 mm). Cesium chloride separations were accomplished by centrifugation at 47,000 r.p.m. (300,000 g) for 10 h at 20°C. After carefully removing the guanidine thiocyanate and cesium chloride, the RNA pellets were washed twice with 100% ethanol, and then resuspended in water and quantitated using UV spectroscopy.

Northern analysis of B cell RNA

B cell RNA was sized fractionated by electrophoresis on a 1% agarose, formaldehyde gel. Each RNA sample was prepared by resuspending 10 μg of total cellular RNA with a buffer consisting of 1× MAE (0.2 M MOPS buffer, pH 7.0, 0.084 M sodium acetate and 5 mM EDTA), 50% deionized formamide and 6.6% formaldehyde. Gels were run for 4–5 h at 70 V and then transferred to a 0.45 μm nitrocellulose membrane (Schleicher & Schuell) by capillary action. After baking the blots at 80°C for 2 h to immobilize the RNA, northern blots were pre-hybridized at 42°C overnight in buffer containing 4 × SSC (0.6 M sodium chloride and 0.06 M sodium citrate), 1% SDS, 5 × Denhardt’s, 50% (v/v) formamide and 100 μg/ml salmon sperm DNA.

Purified cDNA probes (10–50 ng) were radiolabeled with [32P]dCTP using the random primer extension method (22). The ODC and c-myc probes were gifts from Dr Charles Snow (University of Kentucky) and Dr Eric Westin (Virginia Commonwealth University) respectively. Radiolabeled probe was separated from unincorporated [32P]dCTPs using Quick Spin G-25 Sephadex disposable columns (Boehringer Mannheim, Indianapolis, IN). Specific activity of each probe was determined by liquid scintillation spectrometry. Hybridization of filters with appropriate cDNA probes was done at 42°C for 24 h in the buffer described above. After washing at room temperature under low stringency, blots were wrapped in Saran Wrap then exposed to Kodak X-OMAT AR film in the presence of Dupont Cronex Lightning Plus intensifying screens for 12–24 h at ~70°C. Autoradiographs were analyzed by densitometry. Between hybridizations, filters were bathed repeatedly (3–4 times) in boiling water for 10–15 min to remove previously hybridized probes. All Northern blots were probed with a ribosomal probe (23) as a control for RNA.
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Results

**Co-cross-linking CD23 and slg does not alter early B cell signaling events**

Brunswick et al. (1) first reported the potent B cell activation capacity of anti-Ig linked to dextran. As is illustrated in Fig. 1, anti-IgD monoclonal H6/1 linked to DNP-dextran was used to activate purified BALB/c B cells either in the presence or absence of the indicated amount of rat anti-DNP IgE. Previous studies demonstrated that CD23-slg co-cross-linking conditions negatively modulate B cell activation (2). This inhibitory effect was shown to require simultaneous co-cross-linking since cross-linking of CD23 and slg separately using two different dextran conjugates did not have an inhibitory effect on B cell proliferation (2). In addition, when the B cells were from mice that have no CD23 due to gene deletion (24), no inhibitory effects were seen (data not shown), demonstrating that the higher dose of conjugate is not inherently toxic to B cells when IgE is present.

Ligation of surface Ig with antigen or anti-Ig reagents induces a complex series of biochemical and molecular events that lead to B cell proliferation and differentiation. As initially reported by Gold et al. (16), cross-linking of slg results in an activation of protein tyrosine kinases (PTK) which rapidly phosphorylate various substrate proteins. To investigate whether co-cross-linking of IgE and slg influences PTK activity, Triton X-100 soluble proteins isolated from B cells stimulated in the presence of IL-4 with H6/1-[DNP-dextran] alone or with 10 µg/ml rat IgE (anti-DNP) were examined by Western blot analysis. The results shown in Fig. 2 demonstrate that exposure of control B cells with media or 10 µg/ml DNP-specific rat IgE alone induced negligible tyrosine kinase activity. Stimulation of B cells with 5 µg/ml rabbit anti-IgM F(ab')2 fragments (Fig. 2, lane 3), however, caused a significant increase in protein tyrosine phosphorylation. A similar level of phosphorylation is seen when the B cells were stimulated with 3 µg/ml H6/1-[DNP-dextran] (Fig. 2, lane 4) and the presence of anti-DNP IgE in these B cell cultures (Fig. 2, lane 5) did not alter the induction of PTK activity.

These results imply that co-cross-linking CD23 and slg does not affect the ability of tyrosine kinases to phosphorylate their substrates nor does CD23-slg co-cross-linking affect the ability of Ig to transduce growth promoting signals mediated by protein tyrosine phosphorylation. When lower doses of H6/1-[DNP-dextran] (0.03–0.1 µg/ml) were used, we did not observe phosphorylation that was above the media background (data not shown).

Another relatively early event in B cell activation is the up-regulation of ODC activity. ODC is required to enable B cells to progress through the cell cycle (25), and maximal expression of ODC mRNA occurs 6–12 h after entry of the B cell into growth phase. Because co-cross-linking CD23 and slg prevents DNA synthesis and because ODC expression occurs later than protein tyrosine phosphorylation, ODC expression levels were measured as another means of ascertaining the effect of CD23-slg co-cross-linking on early B cell activation events. Total cellular RNA was extracted from B lymphocytes stimulated 8 h in the presence of IL-4 with 3 µg/ml H6/1-[DNP-dextran] ± 10 µg/ml rat IgE anti-DNP and analyzed by Northern blot analysis for expression levels of ODC mRNA. Cross-linking surface IgD with H6/1-[DNP-dextran] stimulated a 7.5-fold increase in ODC mRNA above background. Comparable levels of ODC expression were observed in cultures of B cells stimulated with 3 µg/ml H6/1-[DNP-dextran] and 10 µg/ml rat IgE-anti-DNP (data not shown). Taken together, these results suggest that co-cross-linking CD23 and slg does not impair early B cell activation events, since both total protein tyrosine phosphorylation levels and ODC mRNA levels remain unaltered in B cells subjected to conditions known to inhibit proliferation.

**Co-cross-linking CD23 and slg induces B cell apoptosis**

Although initial PTK activation events are not altered, co-cross-linking CD23 and slg induces striking morphological changes in B lymphocytes. Positive control cultures stimulated
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Fig. 3. Co-cross-linking of CD23 and sIg induces morphology changes suggestive of apoptosis. B cells were stimulated with (A and B) 3 µg/ml Hδα/1–[DNP–dextran] or (C and D) with 3 µg/ml Hδα/1–[DNP–dextran] + 10 µg/ml rat IgE anti-DNP. After 48 h, cells were cytocentrifuged and stained with May–Grunwald Giemsa. ×400 (A and B); ×1000 (C and D). Example blebbing and extruded nuclei are marked in (D) by an arrow and arrowhead respectively.

For 2 days with IL-4 and 3 µg/ml Hδα/1–[DNP–dextran] contain BALB/c B lymphoblasts as shown in Fig. 3(A). Proliferation in these cultures is extensive, and the cells in this culture are enlarged, have a rounded appearance and are in close association with neighboring cells. Under higher magnification, individual cells appear blasted as evidenced by their sparse cytoplasm and large nuclei containing heterogeneous chromatin (Fig. 4B). In sharp contrast, the cultures treated with 3 µg/ml Hδα/1–[DNP–dextran] plus 10 µg/ml rat IgE anti-DNP show less evidence of blast formation, and cellular debris is particularly noticeable (Fig. 3C). Under higher magnification (Fig. 3D) membrane blebbing was apparent (arrow). In some cases, nuclei were being extruded from the cells (arrowhead).

Characteristically, apoptotic cells undergo zesis (membrane ruffling and blebbing), and their nuclei collapse due to condensation and fragmentation of the chromatin. When subjected to CD23–sIg co-cross-linking conditions, cells seemingly undergo morphological alterations characteristic of programmed cell death. Apoptotic cells were also cytocentrifuged then stained with FITC–anti-digoxigenin to determine whether the DNA was fragmented or intact. Cells treated with Hδα/1–[DNP–dextran] and rat IgE anti-DNP incorporate increased levels of FITC–anti-digoxigenin stain, providing further evidence that these cells are undergoing apoptosis (data not shown).

To confirm that co-cross-linking of CD23 and sIg induces B cells to undergo apoptosis, two flow cytometric procedures were utilized to measure the incidence of DNA fragmentation. The advantage of these procedures is that the actual percentage of apoptotic nuclei from B cell cultures can be easily and rapidly measured. Cells stimulated with IL-4 and Hδα/1–[DNP–dextran] in the presence or absence of 10 µg/ml rat IgE were harvested daily over 5 days, lysed in a hypotonic fluorochrome solution containing propidium iodide and the nuclei were then analyzed on a FACSscan. Typically, normal nuclei containing intact diploid DNA demonstrate an increase in forward light scatter with a corresponding decrease in side scatter. Conversely, apoptotic nuclei undergoing DNA fragmentation show a decrease in forward light scatter and an increase in side scatter, thereby allowing the percentage of apoptotic cells to be determined (see Methods). Figure 4(a and b) shows the separation of apoptotic from normal nuclei at day 3 after stimulation (± IgE anti-DNP) using this procedure, and Fig. 4(c) depicts the percentage of apoptotic nuclei detected in B cell cultures over time. The results shown in Fig. 4(c) are representative of two experiments of similar design. In addition, multiple analyses of day 3 nuclei demonstrated <10% variability in the apoptosis values for that time point, further confirming the significance of the differences shown. Cultures sustained with only IL-4 and IgE contained relatively
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Fig. 4. Kinetic analysis of apoptotic nuclei following co-cross-linking of FcRRII and slg. B cells were stimulated with 3 μg/ml Hδα/1−[DNP–dextran] (A and circles) or with 3 μg/ml Hδα/1−[DNP–dextran] + 10 μg/ml rat IgE anti-DNP (B and squares) for 48 h (A and B) or the indicated time in (C). Propidium iodide-stained nuclei were examined by FACS analysis as described in Methods. In (A) apoptotic and normal nuclei are indicated by the arrows labeled ‘1’ and ‘2’ respectively.

Fig. 5. Effect of co-cross-linking CD23 and slg on c-myc mRNA levels. B cells were stimulated in the presence of IL-4 and (open bars) 10 μg/ml rat IgE anti-DNP, (single hatched bars) 3 μg/ml Hδα/1−[DNP–dextran], and (double hatched bars) 3 μg/ml Hδα/1−[DNP–dextran] + 10 μg/ml rat IgE anti-DNP. Stimulation times were 1 h (A) or 8 h (B). Total cellular RNA was probed with the c-myc cDNA probe, stripped and reprobed with rRNA. Results are expressed as signal ratios of c-myc:rRNA to correct for RNA loading differences.

Fewer apoptotic cells than cultures stimulated with Hδα/1−[DNP-dextran] + anti-DNP IgE (data not shown). Thus, we conclude that co-cross-linking CD23 and slg induces a rapid onset of B cell death or apoptosis.

c-myc levels are increased in B cells which are undergoing co-cross-linking of CD23 and slg. The c-myc proto-oncogene is one of several that have been linked to apoptosis (26,27). Entry of cells into the S phase of the cell cycle requires c-myc expression; however, aberrant overexpression of c-myc combined with a block in proliferation actually induces apoptosis (26,27). The expression levels of c-myc mRNA were measured in B cells treated with Hδα/1−[DNP–dextran] alone or in combination with 10 μg/ml DNP-specific rat IgE. Quantitation of c-myc mRNA expression levels was accomplished by Northern analysis of total cellular RNA isolated from B cells cultured for 1 or 8 h, and the results are displayed in Fig. 5. Unstimulated B cells exposed to IL-4 and 10 μg/ml rat IgE expressed negligible levels of c-myc mRNA. Cross-linking surface IgD molecules induced a 100-fold increase in c-myc expression above background; however, co-cross-linking CD23 and slgD induced a more profound up-regulation of c-myc expression. Treatment of B cells with 3 μg/ml Hδα/1−[DNP–dextran] plus 10 μg/ml rat IgE anti-DNP caused c-myc levels to increase 2.75-fold above the positive control, which amounts to a 240-fold increase over background. By 8 h, expression of c-myc was considerably reduced and was only 30- or 14-fold above unstimulated cells for anti-slg and co-cross-linked samples respectively. Thus, between 1 and 8 h, dramatic changes in c-myc expression occurred in B cells subjected to CD23–slg co-cross-linking, suggesting that c-myc was possibly involved in the molecular mechanism of CD23-mediated induction of B cell apoptosis.

We attempted to confirm the importance of c-myc in this apoptosis model by using p53 knockout mice, which have been reported to be resistant to c-myc-induced apoptosis (28). In these animals, co-cross-linking of CD23 and slg
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Fig. 7. Increase in apoptotic cells is not seen with Bcl-2 transgenic B cells. Cells were cultured as in Fig. 6 and subsequently examined for apoptotic cells using the Hoechst staining protocol (21) as described in Methods. Apoptotic cells are boxed and the percentage is indicated.

resulted in a similar decrease in proliferation to that seen with control mice (data not shown), indicating that lack of p53 did not significantly affect apoptosis in this system.

B cells from Bcl-2 transgenic mice are resistant to induction of apoptosis induced by co-cross-linking CD23 and sIg. Aberrant levels of the oncogene bcl-2 have been implicated in a number of models of apoptosis, and an inverse relationship between c-myc and bcl-2 levels has been described previously (reviewed in 29,30). Apoptosis induced by c-myc was found to be inhibited by bcl-2 (31,32). The mice expressing the bcl-2 transgene have a C57BL/6 genetic background (14,15), necessitating the use of a different monoclonal anti-IgD. AF3.33 recognizes a mouse IgD α allotype and was conjugated onto the DNP–dextran backbone in order to stimulate the bcl-2 transgenic B cells. In initial experiments (data not shown), expression levels of CD23 on B cells from the bcl-2 transgenic mice was shown to be essentially identical to control (C57BL/6) animals, both on unstimulated cells and LPS plus IL-4 (33) stimulated B cells. As seen in Fig. 6, B cells from the bcl-2 transgenic mice were stimulated in a dose–response manner by AF3.33–DNP–dextran and addition of anti-DNP IgE had no effect on proliferation. In contrast the expected inhibition of proliferation (2) by co-cross-linking was observed with B cells from control C57BL/6 mice. When the cultures were examined for apoptosis as measured by incorporation of Hoeschs stain (Fig. 7), the percentage of apoptotic cells in bcl-2 transgenic B cell cultures was markedly lower than in control C57BL/6 cultures (8.2 versus 46.7%). This was true regardless of whether CD23 and sIg were co-cross-linked; however, a significant increase in apoptotic cells was observed in control C57BL/6 cultures subjected to co-cross-linking conditions.

Discussion

Bystrom et al. (34) first proposed a theory to suggest that antigen–antibody complexes regulate B cell activation and Ig synthesis. Support for this theory was provided by studies with murine FcγRII, which showed that co-cross-linking of sIg and the FcγRII modulates B cell activation (35,36). Recently, the mechanism for this effect has been suggested to involve dephosphorylation of the immune tyrosine activation motif that is found in Igα and Igβ; this dephosphorylation is mediated by a phosphatase called SHP1 that binds to an immune tyrosine inhibitory motif found in the cytoplasmic domain of FcγRII (37,38).

The studies performed herein were initiated to determine if the CD23 (FcγRII) modulates B cell activation in a manner analogous to the FcγRII. Using the model outlined in Fig. 1, we demonstrated a profound inhibition of B cell proliferation under conditions where the CD23 and sIg were co-cross-linked (2). In contrast to the FcγRII system (4), IL-4 did not reverse the inhibitory effects of CD23. The lack of reversal by IL-4 is also important in that hyper cross-linking of B cell sIg is also known to induce apoptosis (39,40); however, this apoptosis is at least partially reversed by IL-4. The lack of effect of IL-4 in this system indicates that the apoptotic trigger is not simply a result of increased cross-linking due to
increased attachment of the anti-sIg–dextran to the B cell surface via CD23/IgE.

Although B cell proliferation was inhibited by co-cross-linking sIg and CD23, initial B cell activation signals are unaffected. Analysis of PTK activity by anti-phospho-tyrosine Western blot indicated that co-cross-linking of sIg and CD23 did not influence the ability of tyrosine kinases to phosphorylate Triton X-100 soluble cytoplasmic and membrane proteins (Fig. 2). Co-cross-linking sIg and CD23 also did not affect increases in the expression of ODC, an enzyme required for RNA, DNA and protein biosynthesis pathways. Cellular proliferation and differentiation are always accompanied by increases in ODC activity because this enzyme mediates the rate-limiting step in the synthesis of polyamines (41). These results (data not shown) indicate that co-cross-linking sIg and CD23 does not influence the induction of at least one enzyme critical for initiation of DNA biosynthesis.

In this study we demonstrate evidence to suggest that the inability of B cells to successfully self-renew is due to CD23-mediated accelerated induction of programmed cell death or apoptosis. Visual inspection of B cell cultures indicated clear morphological differences between cells stimulated with anti-IgD alone and cells stimulated with H\(_{3}^{3}\)–[DNP–dextran] in the presence of rat IgE anti-DNP. On day 2, B cell cultures treated with H\(_{3}^{3}\)–[DNP–dextran] alone contained numerous B lymphoblasts that were noticeably rounded and enlarged due to an increase in volume. These cells had sparse cytoplasm and pronounced nuclei. In sharp contrast, cultures treated with H\(_{3}^{3}\)–[DNP–dextran] + 10 \(\mu\)g/ml rat IgE anti-DNP contained considerable amounts of cellular debris, and many cells had ruffled membranes and disintegrating nuclei; signs of severe distress. Observation of individual cells from these cultures under higher magnification confirmed the lack of membrane integrity and the dissolution of the nucleus (Fig. 3).

Co-cross-linking CD23 and sIg induces an acceleration of apoptosis in B lymphocytes compared to B cells stimulated with anti-IgD alone. This conclusion comes from analysis of DNA fragmentation both by staining end-labeled DNA and by FACS analysis (20). Only 25% of B cells in cultures stimulated with H\(_{3}^{3}\)–[DNP–dextran] alone were apoptotic. In contrast, almost 50% of the B cells in cultures treated with H\(_{3}^{3}\)–[DNP–dextran] + 10 \(\mu\)g/ml rat IgE anti-DNP for 72 or 96 h contained nuclei with fragmented DNA, as evidenced by their light scatter characteristics. Taken together, these experiments demonstrate that CD23 mediates a negative effect on the B cell response to anti-Ig through the induction of apoptosis.

Expression of certain genes can either suppress or promote cell death by apoptosis. In this study, attempts were made to discern the molecular mechanisms responsible for CD23-mediated induction of apoptosis in B cells by investigating the effect of co-cross-linking CD23 and sIg on c-myc mRNA levels. The results demonstrate that co-cross-linking CD23 and sIg potentiates an early overexpression of c-myc mRNA in B cells. As discussed above, we did not see any changes in ODC levels; several labs have reported that c-myc regulates ODC levels (42,43). A potential reason for not seeing differences in ODC expression relates to the time at which the ODC message was assayed. In addition, these results differ from previous studies showing that cross-linking Fc\(_{\gamma}\)R and sIg inhibited induction of c-myc mRNA expression by anti-IgD (44), again illustrating differences between these two systems. Overall, these studies demonstrate that while the co-cross-linking of sIg and either CD23 or the Fc\(_{\gamma}\)RII both result in inhibition of B cell proliferation, the mechanisms evoking the negative effects are quite different.

The mechanism by which c-myc drives both cell death and cell proliferation is unclear. The product of the c-myc proto-oncogene is a transcriptional factor which is necessary to drive cells into the S phase of the cell cycle, and overexpression of c-myc is frequently associated with neoplasias and is therefore implicated in cell transformation (45). Aberrant c-myc levels have been associated with apoptosis in a number of systems including rat fibroblasts (27), myeloid cells deprived of IL-3 (26), T cell hybridoma apoptosis or immature B cell lines that undergo apoptosis upon cross-linking of the TCR (46) or B cell receptor (47) respectively. Recently, the same kinetics for c-myc induction, i.e. early increase with later suppression, as seen in this study (Fig. 5), was reported for WEHI 231 cells (48), in which apoptosis is triggered by cross-linking the B cell receptor. These same authors also report that NF\(_{\kappa}\)B levels are suppressed in the WEHI 231 cells as well as murine B cells (48,49) undergoing apoptosis. CD40 cross-linking reversed the apoptosis and restored both c-myc and NF\(_{\kappa}\)B levels (48,49). We are currently exploring the effect of CD40 cross-linking in this model and have observed that addition of recombinant CD40L reverses the anti-proliferative effect observed when CD23 and sIg are co-cross-linked (data not shown). We are currently examining the effects on c-myc levels.

Some of the studies found that phosphothiorate antisense c-myc oligonucleotides reverse the apoptosis (47) and, based on this observation, we attempted to reverse CD23-mediated apoptosis using the same oligonucleotides as used in these studies. We found that these c-myc anti-sense oligonucleotides directly stimulated B cell proliferation (data not shown) presumably due to the presence of CpG motifs in the sequence (50), obviating the use of these antisense sequences with mouse B cells. In addition, we were unable to confirm the importance of the aberrant c-myc by using p53 knockout mice. Hermeking and Eick (28) presented evidence that apoptosis induction via c-myc up-regulation was blocked in p53 knockout mice. The decrease in proliferation upon CD23–sIg co-cross-linking with B cells from p53 knockout animals was similar to control mice (data not shown). Thus, the role of the elevated c-myc in this model remains unknown.

A number of apoptosis systems are overridden by the delivery of a second signal (i.e. one provided by growth factors) that encourages cell survival. Such second signals may provoke the activation of the bcl-2 gene. In fact, bcl-2 can reverse the apoptosis induced by CD23–sIg co-cross-linking. B cells from bcl-2 transgenic mice had essentially identical proliferation indices regardless of whether sIg and CD23 co-cross-linked (Fig. 6). In addition, direct measurement of apoptotic cells by FACS analysis revealed no differences in cultures stimulated with AF3.33–[DNP–dextran] alone or in the presence of IgE anti-DNP (Fig. 7). Thus, we conclude that expression of bcl-2 prevents B cell apoptosis induced by co-cross-linking CD23 and sIg.
As mentioned in our initial report (2), these results suggest a means by which antigen-specific IgE can down-regulate additional B cell activation and IgE synthesis. IgE immune complexes may induce B cell apoptosis by co-cross-linking of slg and CD23. This would suggest that animals deficient in CD23 might exhibit enhanced antigen-specific IgE production. In support of this hypothesis, a recent report by Yu et al. (51) suggests that mice deficient in CD23 expression as a result of homologous gene disruption exhibit an increase in antigen-specific IgE responses but not in non-specific IgE responses characteristic of parasitic infections. However, the lack of confirmation of these findings in similar studies (24,52) indicates that additional studies are required to resolve this issue and to determine whether such a model represents a potential target to influence the production of antigen-specific IgE responses in allergic individuals.

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Abbreviations

HS horse serum
ODC ornithine decarboxylase
PTK protein tyrosine kinase

References


