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Bryostatin-1 Specifically Inhibits In Vitro IgE Synthesis

Dania Rabah,* Steve Grant,*† Check Ma,* and Daniel H. Conrad2*

Bryostatin-1, a macrocylic lactone, is an antineoplastic agent that potently activates protein kinase C. Bryostatin-1 (Bryo) had an immunomodulatory effect on murine B cells in that it specifically inhibited IgE production. IgE levels were inhibited in a B cell dose-response curve, whereas IgM and IgG1 were induced by Bryo treatment. Taken together, ELISPOT and surface Ig staining data suggested that Bryo inhibition occurred at the level of class switching. RT-PCR and real time PCR data showed that this inhibition was achieved at an early step in switch recombination, namely, the appearance of Iε germline transcripts. Although Bryo caused a delay in the proliferative response of IL-4/CD40 ligand trimer-stimulated B cells, CFSE studies revealed that the splenic B cells do not express PKCδ, ε, η, and ζ; and the atypical isoenzymes ζ and η (reviewed in Ref. 2). Murine splenic B cells do not express PKCy (3) or PKC6 (4). They express PKC α, β, γ, ξ, and ζ at low levels and abundantly express PKC6 (4). PKC isozymes are differentially expressed during B cell differentiation; e.g., maturation to the plasma cell stage is paralleled by an increase in PKCα and disappearance of the β isofrom (3). PKC is activated by a number of exogenous compounds including phorbol esters such as PMA (5). Another class of PKC activators, structurally unrelated to phorbol esters, is the bryostatins (6, 7). Both PMA and bryostatin-1 (Bryo) activate the classic PKC and novel PKC isozymes.

Bryo is a macrocyclic lactone isolated from the marine bryozoan Bugula neritina (7). It activates and rapidly down-regulates PKC (8–10) and has shown potent antineoplastic properties both in vitro and in vivo (11). Bryo has been shown to induce differentiation in leukemia cells and exert antiproliferative properties in both normal and malignant hematopoietic cells (reviewed in Refs. 12 and 13).

IgE is thought to have evolved as a protective mechanism against parasites, but its production, especially in individuals from developed countries, is associated with allergic responses (14). Based on the direct involvement of IgE production in allergic conditions, several attempts are being made to modulate the synthesis of IgE. To this end, developing a better understanding of the mechanism specifically regulating the IgE isotype is crucial.

This study reports a novel effect of Bryo on in vitro murine and human IgE production models. Namely, IgE production is almost completely ablated, whereas the production of other Igs is either not affected or enhanced. Mechanistic studies indicate that germ-line transcription is impaired, suggesting that reduced switching to IgE is responsible for the effect observed. These findings provide insights into the potential role of PKC in the modulation of IgE production.

Materials and Methods

Abs and reagents

CFSE (Molecular Probes, Eugene, OR) was prepared in DMSO at a concentration of 5 mM as a stock solution and kept at −20°C until used. Nonessential amino acids, 2-ME, sodium pyruvate, HEPES, and PMA were all purchased from Sigma Chemicals (St. Louis, MO). FBS was purchased from HyClone Laboratories (Logan, UT). Baculovirus supernatant containing recombinant murine IL-4 was a gift from Dr. W. Paul (National Institutes of Health, Bethesda, MD); recombinant murine IL-5 was purchased from R&D systems (Minneapolis, MN). Recombinant CD40 ligand trimer (CD40LT) and M15 (mouse IgG1 anti-leucine zipper mAb) were obtained from Immunex (Seattle, WA). Bryo was obtained from the National Cancer Institute (Bethesda, MD). Bryo was reconstituted from powder by preparing a 10−3 M stock in DMSO.

Animals and B cell isolation and cell culture

BALB/c mice were purchased from the National Cancer Institute-Frederick Cancer Research Center (Frederick, MD). All mice used in experiments were between 6 and 14 wk of age. B6 6−/−, B6–129 6−/−, and wild-type controls were generously provided by Dr. A. Dent (Indiana University Medical School, Indianapolis, IN). Single-cell suspension of B lymphocytes isolated from disrupted spleens were negatively selected as described previously (15, 16). Briefly, anti-CD5, anti-CD8 (both from Dr. W. Paul), anti-Thy-1.1 (TIB99), and guinea pig complement (Life Technologies, Gaithersburg, MD) were used to kill T cells. Subsequently, the cells were layered on a discontinuous Percoll gradient, and resting B cells were collected from the 66–70% interface. B cells were then plated at different cell concentrations in 96-well plates (Costar, Cambridge, MA) in a volume of 200 μl B cell medium (RPMI 1640 containing 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 10 mM HEPES buffer, 5 × 10−5 M 2-ME, 100 μg/ml streptomycin, 100 U/ml penicillin, and 10% FBS) and stimulated with 50,000 U/ml IL-4, 5 ng/ml IL-5, 0.1 μg/ml CD40LT, and 0.1 μg/ml M15 at 37°C in a 5% CO2 incubator. These...
activation conditions have been previously shown to be optimal for IgE production (17). In addition, cells were treated on day 0 or the day indicated with 100 nM Bryo. Cultures containing 1% DMSO were used as medium control. To isolate PBL, blood obtained from donors was mixed with an equal volume of sterile PBS, then layered onto Ficoll-Paque (Amersham Pharmacia Biotech, Piscataway, NJ), and centrifuged. The cells at the interface were then collected, washed, and resuspended in RPMI 1640. The optimal concentration of PBL (2 × 10⁶ cells/ml) were costimulated with 200 U/ml human rIL-4 (R&D systems) and 0.5 μg/ml anti-CD40 (BD Pharmingen, San Jose, CA) in human B cell medium (RPMI 1640, 100 μg/ml streptomycin, 100 U/ml penicillin, and 10% FCS from HyClone Laboratories). These stimulation conditions were optimized for IgE production by Prof. Gould’s laboratory (King’s College, London, U.K.). Cell cultures were performed in 96-well plates and were treated with increasing concentrations of Bryo or left untreated. Cells were incubated at 37°C in a 5% CO₂ incubator. Supernatants were harvested at 10–12 days postculture, and IgE levels were assayed by ELISA.

ELISA
IgM, IgG1, and IgE levels were determined by ELISA as previously described (17). Because cells treated with PKC activators lived longer in cultures (data not shown), the absolute value of IgE levels increased with a longer culture period. Therefore, all supernatants were harvested on day 14, which was found to be the optimal time for supernatant collection. Supernatants were then analyzed for IgE as previously reported (17). Briefly, rat anti-mouse IgE mAbs B1E3 and R1E4 were used as the capture and biotinylated secondary Ab, respectively. IgG1 and IgM levels were determined using an unlabeled primary goat anti-mouse Ab at 5 μg/ml and detected using goat anti-mouse class-specific Ab coupled to alkaline phosphatase (Southern Biotechnology Associates, Birmingham, AL). All ELISA were performed in Costar high-binding ELISA plates. For human IgE ELISA, mAbs 7.12 and 4.15 (American Type Culture Collection, Manassas, VA) were used as the capture Abs as previously described (18). Rabbit anti-human-IgE/HRP (DAKO, Carpinteria, CA) was used as the detection Ab. IgM levels were assayed using unlabeled goat anti-human IgM polyclonal Ab at 2 μg/ml as the capture Ab and then detected by HRP-conjugated mouse anti-human IgM Ab (all from Southern Biotechnology) followed by the addition of TMB One Step Substrate (Dako). The reaction was stopped with 0.18 M H₂SO₄ and read at wavelength 450 nm with a SpectraMax ELISA reader.

Proliferation assay
On day 2 postculture or the days indicated, B cells were pulsed using 1 μCi [³H]thymidine/well (ICN Biomedicals, Costa Mesa, CA) for 8 h. Cells were then harvested onto a Unifilter 96 plate (Packard Instrument, Meriden, CT) using a Filtermate 196 plate harvester (Packard), and the incorporation of [³H]thymidine into DNA was measured by reading the plate in a model B9902 TopCount (Packard).

ELISPOT
The protocol used to quantify IgE-producing cells is previously described (19). Briefly, ELISPOT Immulon 4 (Dynex, Chantilly, VA) plates were coated with B1E3 overnight then blocked with a solution of PBS with 5% FBS. Cells isolated on day 5 postculture were added to the plates, incubated for 5 h at 37°C in a 5% CO₂ incubator, and then washed. Spots were detected using biotinylated R1E4, followed by streptavidin-AP and finally 5-bromo-4-chloro-3-indolyl phosphate substrate solution. The number of IgE secreting cells was quantified by counting the number of blue spots per well and multiplying by the dilution factor and was expressed as the number of Ab-forming cells (AFC) per million B cells.

RT-PCR
To determine 1e transcript levels, B cells were stimulated as described above. Total cellular RNA was prepared using TRIZol reagent (Life Technologies) according to manufacturer’s recommended protocol. RNA preparations were quantified by UV spectrophotometry. RT-PCR was performed using the Gene Amp PCR kit (Applied Biosystems, Branchburg, NJ), and the conditions described by Warren and Borton (20). PCR products were detected using [³H]dCTP (3000 Ci/mmol; ICN Biomedicals). A 10% polyacrylamide gel was used to analyze PCR products; the gel was subsequently dried and exposed to a phosphor screen. Quantification of IgE and HGPRT (used as a housekeeping control gene) was performed using a PhosphorImager 445Si (Molecular Dynamics, Sunnyvale, CA) combined with ImageQuant software.

For detection of IgM-Ce postswitch hybrid transcripts, RNA was isolated on day 4 postculture, reverse transcribed as described above, and then amplified using the previously described primer set ImE and CeR (21). PCR products were analyzed on 1.2% agarose and stained with ethidium bromide (Sigma).

Real time PCR
Real time PCR was performed as previously described (22). Briefly, RNA was isolated from the various conditions on different days and reverse transcribed as described above. Real time PCR was performed using the Taqman One-Step RT-PCR Mastermix reagent kit and analyzed on a model 7700 ABI Prism Sequence Detector (Applied Biosystems, Foster City, CA). Ie transcripts were amplified using the primer set IeHF (5’-TGGGACTAATTAAGTTGACTAGG-3’) and IeHR (3’-TGGCCAGACTGTCTATTCGGA-5’) and detected by the Taqman fluorescent probe IeHT (TCAACCGCCTGGGAGCTGTC).

Surface Ig determination
Surface IgE and IgG1 expression was analyzed by fluorescent labeling. For IgE staining, cytoxicidal IgE was removed using the acid stripping protocol. Briefly, cells were harvested, washed, and resuspended in 1 ml acid stripping buffer, pH 4.0 (1.7 g/l sodium acetate, 1.25 g/l NaCl, 0.1 g/l KCl, 1% newborn calf serum). A 60-s incubation, 10 ml PBS/0.1 M HEPES were added to neutralize the acidic solution following by 25 ml Hanks’ medium (Hanks’ BSS, 5% serum, 10 mM HEPES). To block non-specific binding, cells were incubated with 5 μg/ml anti-FcγRII Ab (2.4G2). Stimulated unstained cells were used to correct for autofluorescence. Direct labeling was performed for surface IgE expression by incubating cells with either FITC- or PE-conjugated anti-IgE (Southern Biotechnology) for 45 min at 4°C. Cells stimulated with CD40LT alone, which are unable to switch to IgE, were used as a control for nonspecific IgE binding. IgE expression was detected using an indirect labeling technique; a primary biotinylated rat anti-mouse IgG1 was followed by FITC- or PE-labeled avidin. Nonspecific controls were stained with FITC/PE-avidin only. Immediately before analysis, propidium iodide (PI, 50 μg/ml) and 1% sodium citrate, pH 7.4) was added to the cells to aid in distinguishing dead cells. The viable cell population was gated on forward and side scatter profile as well as PI− population and analyzed on a FACScan (BD Biosciences, San Jose, CA) using Cyclops software (Cytomation, Fort Collins, CO).

CFSE staining and determination of cell division
Resting cells were labeled with CFSE as described previously (23, 24). Briefly, cells were washed and resuspended at 10⁵ cells/ml in PBS containing 0.1% BSA. CFSE was added to a final concentration of 10 μM, and cells were incubated for 10 min at 37°C in the dark. The cells were subsequently washed twice with PBS-BSA and resuspended into complete B cell medium. Labeled cells were then plated in 96-well plates at 2.5 × 10⁵ cells/well; then stimulated with IL-4, CD40LT, and IL-5; and cultured at 37°C in a humid atmosphere containing 5% CO₂. Activated B cells were then harvested at various time postculture, washed, and incubated with PE-labeled anti-IgE mAb (Southern Biotechnology) for 45 min. B cells double-stained with CFSE and PE-anti-IgE were then analyzed on FACScan (BD Biosciences).

Results
Bryo and PMA inhibit IgE production from murine B cells
As previously reported (25), IgE production in vitro was consistently found to be sensitive to B cell dose. IgE levels decreased substantially above 2.5 × 10⁵ cells/well (Fig. 1). Thus, varying B cell doses were stimulated with CD40LT and cytokines in 96-well plates in the presence or absence of Bryo. Interestingly, treatment with Bryo led to a dramatic inhibition of IgE production as evidenced by ELISA (Fig. 1A). Identical results were observed for BALB/c and C57BL/6 mice (data not shown). This inhibition was selective for the IgE isotype; both IgM and IgG1 production were enhanced after treatment with Bryo (Fig. 1, B and C). This effect was not unique to Bryo, given that PMA (the classic PKC activator) showed a similar specific inhibition of IgE production (data not shown).
Time course of Bryo-mediated IgE inhibition

To determine how long Bryo had to be present to observe the inhibitory effect on IgE production, Bryo was added on different days after culture initiation. As shown in Fig. 2, Bryo had a maximal effect on IgE production when added up to day 2 postculture. A modest inhibition by Bryo could be achieved even 5 days after culture initiation. The cell concentration depicted in Fig. 2 (1 × 10^3 cells/well) is representative of B cell doses ranging from 250 to 2.5 × 10^3 cells/well, where IgE levels are not sensitive to cell density in culture wells.

Inhibition is occurring at the level of the germline e transcription

Production of IgE can be dissected into different critical steps starting with switching. The first step in switching involves transcription of the H chain Ig genes in their germline configuration. Subsequently, the active region is targeted for switch recombination and produces the mature transcript. Afterward, the cell goes through differentiation to ultimately produce IgE-producing plasma cells (reviewed in Ref. 26).

To determine the level at which IgE inhibition is occurring, the ability of murine B cells to differentiate in the presence of Bryo was determined. To this end, B cells treated with Bryo were examined by ELISPOT to determine whether reduction in IgE production correlated with a reduced number of IgE Ab-forming cells (IgE AFC). Fig. 3A shows that production of IgE-forming cells, the terminal stage of B cell differentiation, is greatly impaired after treatment with Bryo. These data indicate that Bryo causes a decrease in the number of IgE AFC rather than a decrease in IgE production per cell, suggesting a possible effect on class switching.

Next, the effect of Bryo on surface Ig expression was quantitated to determine whether the drug is preventing class switching to IgE. Fig. 3B shows that surface IgE expression is strongly inhibited by Bryo. In contrast, IgG1 expression is higher than control levels, especially on day 4 postculture. These observations are consistent with the levels of Ig secreted by the cells (Fig. 1). On day 5, the proportion of IgG1-positive cells decreased in both control and Bryo-treated cells (20.2% vs 1.4% in control cells; 47.4% vs 20.7% in Bryo-treated cells on days 4 and 5, respectively). This could be explained by the loss of surface Ig expression as the cells differentiate to the plasma cell stage. Control IgG1 expression may also be lost as B cells switch to IgE, because the majority of IgE-expressing cells are generated by sequential switching from IgM to IgG1 via IgG2 (27). This interpretation is supported by the presence of a population of B cells that is double-positive for IgG1 and IgE on day 4 and the increase in the percentage of these cells and IgE-positive cells on day 5 (Fig. 3B, left). In contrast with control cells in which switching to IgG1 is not an end stage of differentiation, Bryo-treated cells fail to switch to IgE leading to the accumulation of IgG1 (Figs. 1C and 3B).
After class switching, the Iε exon, its promoter, and its enhancer are still present and active. Therefore, transcription is usually initiated at the Iε promoter and terminated 3′ of the switched Cε gene. After processing, the resulting transcript is composed of the Iε exon spliced to the Cε exon. Thus, the detection of Iε-Cε transcripts by RT-PCR is generally accepted as an indicator of class switching to IgE (28). As shown in Fig. 3E, hybrid Iε-Cε transcripts were inhibited in Bryo-treated cells indicating that Bryo impairs class switching to the IgE isotype.

The inhibition was traced back to an early and essential step in IgE switching, i.e., germline ε transcription. RNA isolated from B cells stimulated in the presence or absence of Bryo, was screened for germline ε transcripts (Iε). Stimulation with CD40LT and IL-4, as expected, activated Iε transcription. In contrast, treatment with Bryo consistently inhibited the appearance of Iε transcripts (Fig. 3C). The quantitative technique, real time PCR, was used to confirm the hypothesis that Bryo inhibits germline ε transcription. Fig. 3D shows an analysis of these data normalized for the levels of a housekeeping gene (mouse β-actin). The data provide evidence that the germline transcription is dramatically reduced on Bryo treatment, thereby inhibiting an early requisite for IgE switching (Fig. 3D).

**FIGURE 3.** Bryo inhibits class switching to the IgE isotype. B cells were stimulated with CD40LT, IL-4, and IL-5; treated with control medium or 100 nM Bryo and plated at increasing B cell concentration per well ranging from 250 to 10⁴ cells/well (A) or at 1 × 10⁴ cells/well (B–E). A, Cells were then used in an ELISPOT assay as described in Materials and Methods. Error bars represent ±1 SE. Shown is one of three independent experiments. B, Cells were harvested on the days indicated and were double stained with FITC anti-IgE and biotinylated anti-IgG1 followed by streptavidin-PE. The bivariate dot plot is representative of two experiments performed. C, RNA was isolated on the days indicated and RT-PCR products were amplified as described in Materials and Methods. Lanes 1 and 10, Negative control (no RNA); lanes 2, 3, 6, and 7, RNA isolated on day 3; lanes 4, 5, 8, and 9. RNA isolated on day 5; lanes 2, 4, 6, and 8, control; lanes 3, 5, 7, and 9, Bryo. D, Real time PCR analysis of Iε transcription; each RNA sample, isolated on the days indicated, was analyzed in triplicate at two RNA concentrations. Error bars represent ±1 SE. This experiment is representative of two performed. E, RT-PCR of Iε-Cε postswitch hybrid transcripts.

Bryo-mediated IgE inhibition is independent of the delay in cell division

In addition to inhibiting switching to IgE, Bryo-reduced IL-4 and CD40LT induced proliferation especially at earlier time points after culture initiation (days 2 and 3) (Fig. 4). However, by day 4, Bryo and control cells exhibited similar proliferative responses. B cells need more rounds of division to differentiate to IgE-producing cells than to cells producing other isotypes. For instance, switching to IgG1 requires three rounds of divisions, whereas IgE requires five divisions (24). Because IgE inhibition coincided with a reduction of proliferation, particularly on the days preceding class switching, we investigated the possibility that Bryo is inhibiting B cells from achieving the required number of divisions for IgE production. CFSE, a cell-permeant fluorescein-based dye, was used to track individual cell divisions by the sequential halving of the dye content (23). Labeled B cells were stimulated with CD40LT, IL-4, and IL-5 in the presence or absence of Bryo. B cell treatment with IL-4 alone was used to determine the fluorescence of zero division cells (D₀). These cells remain viable but do not divide. Dₘₐₓ refers to the maximum number of divisions observed at a particular time point. Fig. 5 shows the time course of
Bryo reduces proliferation of murine B cells in vitro at early time points postculture. B cells were stimulated with CD40LT, IL-4, and IL-5; treated with control medium or 100 nM Bryo and plated at 5 × 10^3 cells/well. Cells are pulsed with [3H]thymidine on the days indicated for 8 h, and proliferation was measured as cpm incorporated. Error bars represent ± SE. This experiment is representative of three performed.

Bryo delays B cell division. B cells (2.5 × 10^5 cells/well) were stained with CFSE or left unstained (dotted line); then stimulated with CD40LT, IL-4, and IL-5 and treated with control medium (thick black line); or 100 nM Bryo (thick gray line). Cells stimulated with IL-4 (thin black line) represent nondividing (D0) cells. Cells were harvested on the days indicated, and cell division was analyzed by flow cytometry. This experiment is representative of four experiments conducted.

Bryo inhibits IgE production from human B cells in vitro

Human PBL were stimulated with predetermined optimal concentrations of human rIL-4 and anti-CD40 and were treated with increasing concentration of Bryo. As shown in Fig. 8, as little as 0.1 nM Bryo led to a 60% inhibition of IgE production. Interestingly, when compared with their murine counterpart (Fig. 8, inset), human B cells were 10-fold more sensitive to Bryo-mediated IgE inhibition. This inhibition appeared to be selective to the IgE isotype because IgM levels were not affected at subnanomolar concentrations of Bryo and were consistently induced at higher doses (Fig. 8B). Similar results were obtained using purified B cells (data not shown). Control IgG4 levels were undetectable by ELISA, precluding the analysis of the effect of Bryo on this isotype. Collectively, these data indicate that Bryo actions are operative in both murine and human systems.

Discussion

Allergic conditions constitute a group of diseases of rising importance in developed countries. IgE is closely associated with hypersensitive responses; therefore, recent efforts have been focused on blocking IgE secretion and/or synthesis.
FIGURE 6. Bryo inhibits switching to IgE independently of the number of cell divisions. Cells (2.5 \times 10^3 \text{cells/well}) stimulated and stained with CFSE were harvested on the days indicated and stained with PE anti-IgE. A, Dot plot of IgE expression by CFSE-labeled cells treated with control medium (left) or Bryo (right). The vertical dotted lines show positions of the cell divisions. B, The data in A were replotted as a kinetic analysis. Each division was gated on and percent PE^+ events were calculated. This experiment is one of three experiments performed.
In this study, treatment with Bryo showed a selective inhibition of IgE production (Fig. 1A). This inhibition was selective as both IgM and IgG1 levels were enhanced by Bryo (Fig. 1, B and C). Similar results were obtained with PMA, a classic activator of PKC (data not shown). Attempts to address the direct involvement of the PKC pathway in this inhibition using selective PKC inhibitors were unsuccessful. Both chelerythrine chloride and bisindolylmaleimide (GF109203X) were toxic to B cells (data not shown) consistent with the fact that PKC inhibitors are potent inducers of apoptosis especially in hemopoietic cells (32). Given the recent discovery of phorbol ester receptors that bind PMA or 1,2-diacylglycerol but lack kinase activity (reviewed in Ref. 33), it is possible that IgE inhibition is mediated by a PKC-independent pathway.

Data from ELISA (Fig. 1) and ELISPOT (Fig. 3A) assays could be interpreted to signify that B cells fail to terminally differentiate to IgE-forming cells in the presence of Bryo. The fact that the time course of Bryo inhibition was maximal up to day 2 postculture (Fig. 2), when the switching process starts, points to a major defect in switching rather than maturation to IgE-secreting plasma cells. This is further supported by flow cytometry analyses showing inhibition of sIgE expression in Bryo-treated cells (Figs. 3B and 6A) and a lack of postswitch transcription (Fig. 3E). The inhibition was manifested at the level of germline transcription, which is a critical step in switch recombination (Fig. 3, C and D). However, an effect on other aspects of differentiation cannot be excluded because Bryo continued to show some degree of inhibition on IgE levels as late as day 5 postculture, after switching had occurred (Fig. 2).

In addition to its selective effect on IgE production, Bryo reduced the proliferative response of murine B cells on days 2 and 3 postculture (Fig. 4). Because proliferation is intimately related to isotype switching, one explanation for the results could be an insufficient number of cell divisions. Isotype switching has been linked to cell division by a number of reports, and B cells were found to require a minimum number of divisions before switching to the various isotypes (29). Three rounds of divisions are needed for switching to IgG1 vs five divisions for switching to IgE (24). By reducing proliferation, Bryo would provide a selective advantage for IgG1 switching while failing to meet the required number of divisions for IgE switching. This possibility appeared plausible based on the finding that cell division regulated the levels of germline transcripts (34), the step in which Bryo exerted its inhibitory effect. CFSE labeling, however, refuted this hypothesis and provided evidence that proliferation was only delayed in Bryo-treated cells (Fig. 5). Despite the delay, cells treated with Bryo are not at a disadvantage for IgE switching because they remain viable for a longer period of time in culture than do untreated cells (data not shown). Nevertheless, Bryo inhibited switching to IgE at all cell division cycles (Fig. 6). Interestingly, in our system the threshold division number for switching to IgE is lower than values previously reported (Ref. 24; Fig. 6). This apparent discrepancy may be explained by the use of a higher concentration of rIL-4 (50,000 U/ml) in our experiments. This is consistent with data from Hodgkin et al. (24) in which the number of divisions preceding isotype switching to IgG1 decreased with increasing IL-4 concentration.

After ensuring that the delay in proliferation did not represent the mechanism by which Bryo regulates switch recombination, the germline e promoter regulation was examined. This promoter provides a site where both negative and positive signals integrate and where Bryo might be exerting its selective inhibitory effect. Optimal activation of this promoter is achieved by cooperative synergy...
between several transcription factors (35). In response to IL-4 stimulation, STAT6 binds to its target DNA (36). CD40 signaling activates NF-κB (37), which then interacts with both STAT6 and RNA polymerase to drive transcription from the Ιε promoter (38, 39). This multicomponent complex is believed to be coordinated by a B cell-specific factor, B cell-specific activator protein (40). Bryo could be interfering with any of the cis-controlling elements that regulate Ιε transcription, particularly STAT6 and NF-κB. Because these two transcription factors are also important for IgG1 production and the latter is enhanced by Bryo, this possibility seems unlikely.

Specific negative signals acting on this promoter include high mobility protein (HMG-I(Y)) and the transcription repressor BCL-6. HMG-I(Y) binds to the transcription initiation site, thereby inhibiting transcription (41, 42), whereas BCL-6 inhibits transcription by competing with STAT6 for its binding site (31). As shown in Fig. 7, BCL-6+/−B cells were as susceptible as wild-type cells to Bryo-mediated inhibition of IgE production, suggesting that the inhibition of IgE switching by Bryo is not mediated through BCL-6.

HMG-I(Y) is another important negative regulator of IgE production. HMG-I(Y) belongs to a family of nonhistone high mobility group protein that bind to the minor groove of DNA clusters (43–45). In response to IL-4 downstream of insulin receptor substrate-1 signaling, HMG-I(Y) is serine phosphorylated (41, 46). Phosphorylation of HMG-I(Y) results in an attenuation of binding to its target DNA, thus allowing transcription to resume (41). HMG-I proteins can be phosphorylated by serine/threonine kinases such as cdk2 kinase (47, 48) and casein kinase II (49), and recently HMG-I(Y) is shown to be a substrate of PKC. PKC-catalyzed phosphorylation of HMG-I(Y) is another important negative regulator of IgE production. HMG-I(Y) belongs to the nonhistone high mobility group protein that bind to the minor groove of DNA clusters (43–45). In response to IL-4 downstream of insulin receptor substrate-1 signaling, HMG-I(Y) is serine phosphorylated (41, 46). Phosphorylation of HMG-I(Y) results in an attenuation of binding to its target DNA, thus allowing transcription to resume (41).

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