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# Activation of the Aryl Hydrocarbon Receptor/Transcription Factor and Bone Marrow Stromal Cell-Dependent PreB Cell Apoptosis[1]

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In the absence of known endogenous ligands, investigators have exploited ubiquitous environmental pollutants, including polycyclic aromatic hydrocarbons, to gain insight into the physiologic functions of the aryl hydrocarbon (dioxin) receptor/transcription factor (AhR). AhR ligands induce cell transformation and steroid-like immunosuppression, suggesting a role for the AhR in regulation of cell growth and/or function. However, mechanisms through which the AhR influences cells in general and lymphocytes in particular remain unresolved. A murine model of B cell development was created to: 1) examine a role for the AhR in immunosuppression; 2) define mechanisms of AhR ligand immunosuppression; 3) characterize AhR expression in preB cells, in bone marrow stromal cells that support preB cells, or in primary bone marrow B cells; and 4) determine if AhR ligands suppress lymphopoiesis by acting directly on preB cells or indirectly via the microenvironment, as represented by bone marrow stromal cells. Results indicate that: 1) low doses ($\geq 10^{-8}$ M) of the prototypic AhR ligand, 7,12-dimethylbenz[a]anthracene (DMBA), induce preB cell apoptosis in 12 to 24 h; 2) $\alpha$-naphthoflavone, an AhR and cytochrome P-450 inhibitor, blocks DMBA-induced apoptosis; 3) AhR mRNA and functional AhR protein are expressed at high levels in bone marrow stromal cells (little or no AhR is present in preB cell lines), and 4) preB cells maintained in rIL-7 do not undergo DMBA-induced apoptosis unless cultured with stromal cells. Results underscore the regulatory role played by bone marrow stromal cells in lymphopoiesis and support the hypothesis that the AhR effects immunosuppression by inducing stromal cells to deliver a death signal to lymphocytes. *The Journal of Immunology,* 1997, 158: 2165–2173.

T he 8S aryl hydrocarbon (dioxin) receptor (AhR)[4] is a cytosolic protein that is converted to a nuclear transcription factor upon activation (1–3). Its ability to bind ligand is dependent on association with 90-kDa heat shock protein and its capacity to bind xenobiotic-specific DNA response elements (XRE) and induce gene transcription is dependent on dimerization with an accessory molecule, the AhR nuclear translocator protein (Arnt) (4, 5). While endogenous AhR ligands have not been adequately defined and the "physiologic" function of the AhR remains to be determined, considerable interest in this receptor has been generated by the demonstration that a wide variety of common environmental pollutants, including polycyclic aromatic hydrocarbons (PAH), polychlorinated biphenyls, and halogenated aromatic hydrocarbons (e.g., dioxins), induce AhR translocation, XRE binding, and an array of molecular and cellular responses. These responses include, but are not limited to, induction of PAH-specific cytochrome P-450 genes (1, 6); activation of c-Ha-*ras*, c-*myc*, and c-*erb*-A proto-oncogenes (7, 8); modulation of glucocorticoid, epidermal growth factor, and estrogen and progesterone receptors (9, 10); and induction of cyclin-dependent and protein tyrosine kinases (11, 12). Like the family of steroid receptors, the AhR associates with hsp90, converts to a transcription factor, which induces enzyme and growth factor genes, and is involved in immunosuppression and immunotoxicity. These observations suggest a role for the AhR in cell growth and function.

Similarly, the ability of these exogenous AhR ligands to modify lymphocyte signaling (13, 14); to induce IL-1$\beta$, TGF-$\alpha$, and TGF-$\beta$ gene transcription (15–17); and to modulate both T and B cell responses (6, 18–28) suggests that the AhR may play a role in lymphocyte function or development. Recent evidence that lymphocyte development is impaired in AhR gene knock-out mice supports this hypothesis (29).

Despite these studies, it is not known whether the AhR mediates its immunomodulatory effects via direct signaling in lymphocytes or by indirect effects on the supporting lymphocyte microenvironment. Indeed, studies with immature lymphocyte populations (30, 31) and with mature T cell populations and clones (32) suggest the latter mechanism.

Reasoning that developing biologic systems are exquisitely sensitive to environmental chemicals (33) and that the AhR may play a role in lymphocyte development (29), we developed an in vitro

model of murine B lymphopoiesis to determine the biologic consequences of AhR activation. Particular attention was focused on the role of the microenvironment in regulating lymphopoiesis. In the system used, cultures of cloned bone marrow stromal cells and a stromal cell-dependent early preB cell line were exposed to the prototypic AhR ligand and immunosuppressant (27), 7,12-dimethylbenz[a]anthracene (DMBA). Studies represented here assessed the effects of relatively low AhR ligand concentrations on the immature B cell microenvironment, characterized the expression and function of AhR in bone marrow stromal cells and in preB cells, and determined if AhR-dependent modulation of lymphocyte development results from direct interactions between an AhR ligand and lymphocytes or from signals delivered to immature B cells via AhR$^+$ stromal cells.

# Materials and Methods

## Derivation and DMBA treatment of preB cell lines

Murine bone marrow cultures that support the growth of immature B lymphocytes were prepared from C57BL/6 bone marrow exactly as described (34). After 4 wk of culture, stromal cell-adherent cells (>95% B220$^+$ by flow cytometric analysis) were gently washed free of plate-adherent stromal cells and transferred to confluent monolayers of a cloned bone marrow stromal cell line, BMS2, shown to support preB lymphocyte growth (35). Transferred lymphocytes readily adhered to BMS2 cells and lymphocyte growth was evident within 3 wk. Resulting cell lines, one of which is referred to as BU-11, were maintained in RPMI 1640 medium containing 5 to 10% FCS (Life Technologies, Grand Island, NY), 2 mM L-glutamine (Life Technologies), 50 U/ml penicillin/streptomycin (Life Technologies), and 5 × 10$^{-5}$ M 2-ME (Mallinckrodt, Paris, KY). Cell lines were fed every 3 days and split approximately 1:8 every 4 days to maintain log growth. The BU-11 cell line has been maintained for over 1 yr with no change in surface phenotype or growth characteristics. Since murine AhR polymorphisms affecting AhR expression and function have been reported, it should be noted that both BU-11 and BMS2 cells were derived from mice (C57BL/6; AhR$^{b-1}$ and [B6D2]F$_1$; AhR$^{b-1}$/AhR$^d$, respectively) expressing relatively high levels of high affinity AhR (36). To assess apoptosis, cultures were treated with vehicle (acetone) or various concentrations of DMBA dissolved in acetone (final vehicle concentration, 0.1%) in duplicate wells. In some experiments, α-naphthoflavone (α-NF) dissolved in acetone was added (final vehicle concentration, 0.1%). At various points thereafter BU-11 cells from duplicate wells were pooled and assayed for apoptosis. Vehicle had no effect on cultures. Similar results were obtained with all lines tested.

## Enrichment of bone marrow B cells

Bone marrow cells were expunged from the femurs of C57BL/6 mice. Monocytes and stromal cells were depleted by incubating cells in tissue culture plates for 3 h at 37°C. Nonadherent cells were then incubated for 1 hr at 4°C on petri dishes coated with 10 μg/ml anti-CD45/B220 Ab (PharMingen, San Diego, CA). Adherent cells were removed following a 5-min incubation with Versene (Sigma Chemical Co., St. Louis, MO). Populations enriched for bone marrow B cells were >85% B220$^+$ as assessed by flow cytometry.

## Fluorescence analyses

For surface Ag phenotyping, BU-11 cells were harvested by gently washing cultures with medium. Cells were incubated for 40 min on ice with the following Abs (PharMingen): FITC-anti-CD45/B220 (clone RA3-6B2, rat IgG2a), phycoerythrin-anti-CD43 (clone S7, rat IgG2A), phycoerythrin-anti-mouse IgM (clone LO-MA, rat IgG2b), or isotype controls labeled with an appropriate fluorochrome. Cells were washed, fixed in 1% formaldehyde, and analyzed in a Becton Dickinson (Mountain View, CA) FACscan flow cytometer. Dead cells were gated out of analyses based on forward and side light scatter parameters.

Quantitation of apoptotic cells was performed as previously described (19, 37). Cells were washed in 4°C PBS, pelleted, and resuspended in 0.5 ml of hypotonic fluorochrome solution containing 50 μg/ml propidium iodide (PI) (Sigma), 0.1% sodium citrate, and 0.1% Triton X-100 (Sigma). Cells undergoing DNA fragmentation and apoptosis were shown to be those in which PI fluorescence was weaker than the typical G$_0$-G$_1$ cell cycle peak. For analysis of cell morphology, cells were resuspended in PBS containing 10% FCS.

## DNA gels

Cells (10$^6$) were washed and resuspended in 4°C Tris (10 mM)/EDTA (1 mM) buffer containing 0.2% Triton X-100. Debris was pelleted and supernatant transferred to a fresh tube. After addition of 35 μl of 3 M sodium acetate, DNA was extracted by phenol-chloroform extraction. Fragmented DNA in supernatants was precipitated with ethanol, pelleted, rinsed with 4°C ethanol, dried, and resuspended in TE buffer. For gel electrophoresis, samples were added to loading buffer consisting of 40% sucrose in TE buffer, 1% SDS (Sigma), bromphenol blue, and 2.5 μg/ml RNase (Life Technologies) and loaded into dry wells of a 3.5% NuSieve agarose gel (FMC Bioproducts, Rockland, ME) in Tris acetate buffer. Gels were run at 50 V for 2 h and stained with ethidium bromide.

## AhR immunoblotting

BU-11 cells were gently washed off BMS2 cultures and transferred to new culture wells for 3 h before harvest to minimize contamination with plate-adherent BMS2 cells. BU-11 populations contained <1% BMS2 cells as assessed initially by flow cytometry (forward and light scatter parameters, B220 expression) and later by reculturing an aliquot for 2 days and counting the number of large, fibroblast-like BMS2 cells. BMS2 cells were lifted from plates by a 3-min treatment with 0.25% trypsin containing 1 mM EDTA · 4Na (Life Technologies), washed twice in 4°C PBS, resuspended in lysing buffer (1% Triton X-100, 150 mM NaCl, 25 mM Tris/HCl, 1 μg/ml aprotenin, 10 μg/ml leupeptin, 1 mM EDTA, 50 mM NaF, 1 mM o-vanadate, and 1 mM PMSF) and centrifuged for 15 min at 15,000 × g. Protein concentrations in supernatants were measured with a bicichoninic acid protein assay reagent kit (Pierce Chemical Co., Rockford, IL). Samples were diluted in Laemmli buffer and loaded into 7.5% SDS polyacrylamide gels. Electrophoresis was carried out at 150 V for 1 h. Proteins were transferred from gels to nitrocellulose filters (Bio-Rad, Hercules, CA) at 150 V for 1 h or at 30 V overnight. Efficiency of transfer was monitored by staining proteins with 0.1% Ponceau S (w/v) in 5% acetic acid (v/v) solution (Sigma). Ponceau S was washed out with double distilled water followed by TBST buffer (20 mM Tris, 0.5 M NaCl, 0.03% Tween 20, pH 7.5). Filters were blocked with TBST buffer containing 5% dry milk, washed twice for 5 min in TBST, and incubated with anti-AhR mAb Rpt1 (38) at a 1/10,000 dilution for 1 h at room temperature. Filters were washed three times with TBST and incubated for 1 h at room temperature with a 1:6000 dilution of horseradish peroxidase-goat anti-mouse Ab (Sigma). Filters were washed twice and developed by chemiluminescence (DuPont NEN Research Products Co., Boston, MA).

## Nuclear protein isolation

Seventy percent confluent BMS2 or Hepa-1 cells were treated for 3 min with 0.25% trypsin, 1 mM EDTA · 4 Na (Life Technologies), pelleted, washed twice in 4°C PBS buffer, and resuspended in 1 ml P$_{10}$EG buffer (10% glycerol, 8.4 M KH$_2$PO$_4$ · 3H$_2$O, and 10 mM EDTA, pH 7.4) plus 0.2% Triton X-100. Cell suspensions were rigorously pipetted and nuclei centrifuged for 12 min at 6000 × g. The quality of nuclei preparations was monitored by phase-contrast microscopy. Nuclei were washed twice with P$_{10}$EG and resuspended in lysing buffer and protein prepared for immunoblotting as described above.

## In situ hybridization

$^{35}$S-radiolabeled AhR riboprobes were generated using T7 (sense) and SP6 (antisense) promoters with linearized Xba and HindIII digests of pcDNA-AhR murine AhR cDNA as template. Cultures of BU-11 preB cells maintained on BMS2 monolayers on glass slides were fixed with 4% paraformaldehyde, dehydrated, and hybridized for 18 h at 52°C with either sense or antisense riboprobe. Slides were washed and autoradiographs exposed for 90 days.

## Quantitative RT-PCR for AhR mRNA

A PCR "competitive mimic" was generated as an internal standard as described (39). The AhR mimic contained 400 nucleotides of φX174 sequence enclosed by the same primers as above. The mimic was generated by PCR with hybrid primers TCATGCGGGAAGCCTTCAAGAAG and AAGGGAGGCATCTGGCTATGATG (part φX174 and part AhR primers; see below) and φX DNA template. The amplified band was then reamplified with AhR primers and cloned by A/T cloning into the vector pGEM-T (Promega Co., Madison, WI).

Whole cell RNA was extracted from 5 × 10$^6$ cells using "RNAzol" as described by the manufacturer (Leedo Medical Laboratories, Houston, TX). BMS2 cells were prepared as described above and the adherent layer of cells was lysed directly. BU-11 cells were prepared as described above with the exception that they were cultured for 7 days in rIL-7 and passed three to four
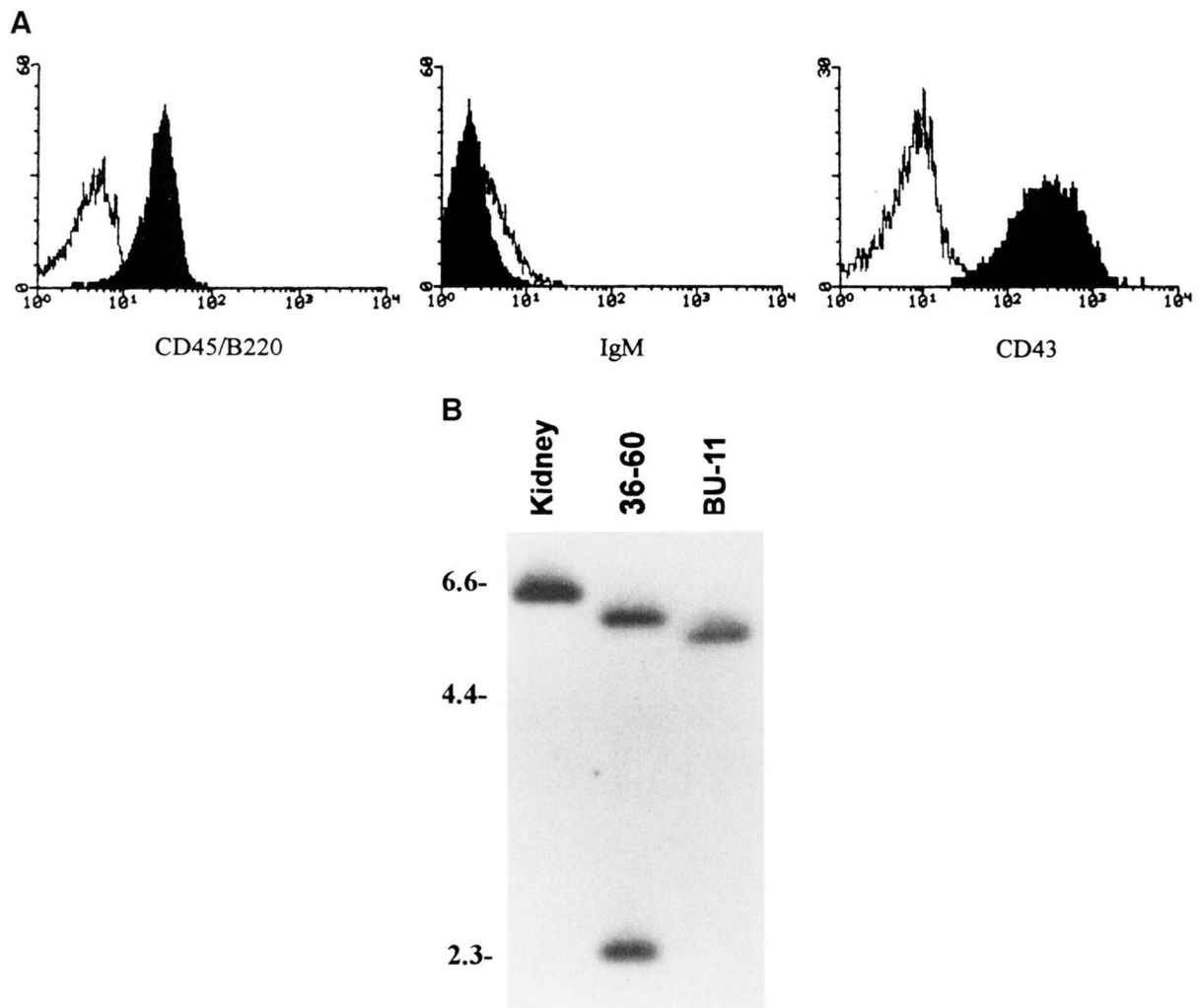
**FIGURE 1.** Characterization of the BU-11 cell line. *A*, BU-11 cells were harvested from adherent monolayers of BMS2 cells (<1% BMS2 cell contamination) and stained with fluorochrome-labeled isotype control Abs or Abs specific for CD45/B220, CD43, or surface IgM. (*B*), DNA was extracted from an aliquot of BU-11 cells, murine kidney cells, or the 36-60 B cell hybridoma; electrophoresed; transferred to nylon filters; and blotted with a JH-specific probe.

times to eliminate BMS2 cells. BU-11 cells were centrifuged and the pellet was lysed with RNAzol. The integrity of RNA samples was assayed by electrophoresis in 1.5% 10 mM phosphate agarose gels prior to RT-PCR.

RT-PCR to detect AhR mRNA in 5-$\mu$g samples was performed as described by the manufacturer (SuperScript preamplification system, Life Technologies) with MgCl$_2$ concentration adjusted to 2.5 mM to maximize specific signal and using the following AhR primers: CTGGCAAT GAATTTCCAAGGGAGG and CTTTCTCCAGTCTTAATCATGCG. Primers were chosen to enclose the sequence that contains the putative murine AhR ligand-binding domain (2, 3). AhR mRNA was reverse transcribed and aliquots were added to known titered amounts of the linearized mimic cDNA. Amplified DNA (10 $\mu$l of 100 $\mu$l; 35 cycles) was electrophoresed through 3% gels (3:1 NuSieve:LE agarose, FMC Bioproducts) and DNA visualized by ethidium bromide. AhR cDNA was assumed to equal the amount of mimic added when the density of the AhR band equaled that of the mimic (39).

### Southern blot analysis of heavy chain rearrangements

Procedures for Southern blotting and hybridization using the JH probe "P2" have been previously described in detail (40) with the exceptions that charged nylon filters were used and that the DNA transfer was done using alkaline transfer as described by the manufacturer (GeneScreen Plus, Du-Pont NEN Research Products.

## Results

### DMBA induces apoptosis in a preB cell line

Flow cytometric analyses indicated that BU-11 cells express B220/CD45 and CD43 but not IgM surface Ags (Fig. 1*A*). This phenotype is consistent with that expressed by primary proB cells (41). However, Southern blotting analysis demonstrated an Ig heavy chain gene rearrangement in BU-11 cells (Fig. 1*B*). Therefore, the BU-11 line is pauciclonal or monoclonal and likely represents B cells at the transition point between pro- and preB cells. For simplicity, we refer to them as early preB cells.

To determine the effects of activating the AhR in bone marrow stromal/preB cell cultures, vehicle, or the prototypic AhR ligand, DMBA (10$^{-4}$ M) was added to cultures of BU-11 cells growing on BMS2 stromal cells. Eighteen hours later cells were harvested and the proportion undergoing apoptosis was quantitated by DNA staining with PI and by flow cytometric analyses of cell morphology (19). Data from 1 experiment (from >25 total) are presented in Figure 2*A*. Relatively few (2%) vehicle-treated cells exhibited a dull PI staining pattern characteristic of cells undergoing apoptosis. Similarly, few

**A**



**B**
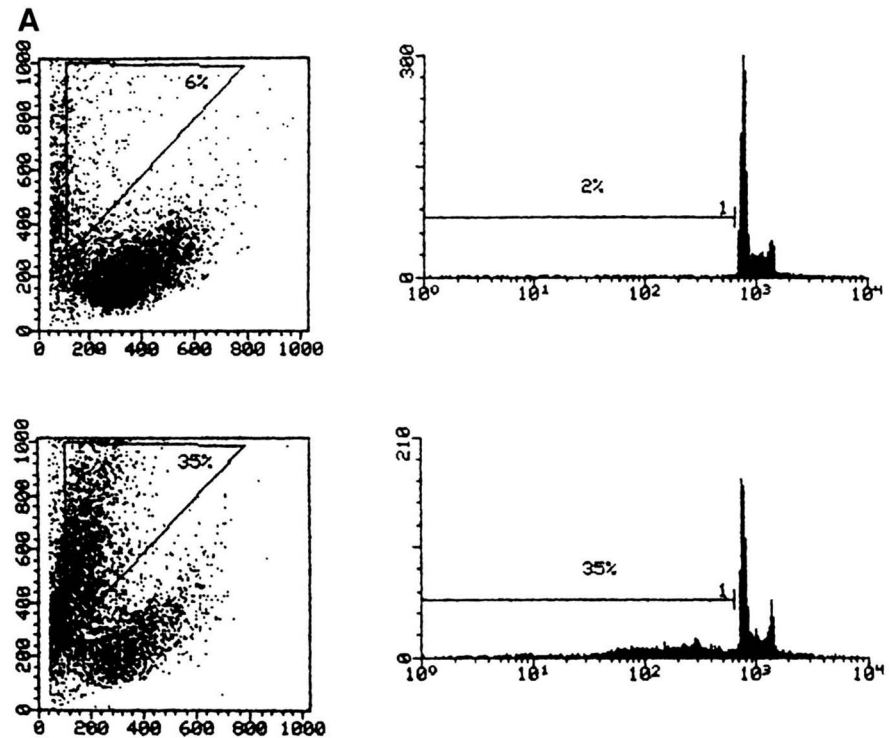


**FIGURE 2.** DMBA induces apoptosis in BU-11 cells. Vehicle (0.1%) or DMBA ($10^{-4}$ M) was added to BU-11 cells in duplicate wells. *A*, Wells were pooled 18 h later and separated into two aliquots. One aliquot was treated with PI in hypotonic buffer for DNA analyses (histograms) and the other aliquot was resuspended in PBS for forward (size) and side (granularity) light scatter analyses (dot-plots). Apoptotic cells stain poorly with PI and are relatively small and granular (enclosed region of each dot-plot). *B*, BU-11 cells were harvested 12 to 24 h after DMBA exposure, DNA extract, and electrophoresed in 3.5% agarose gels.
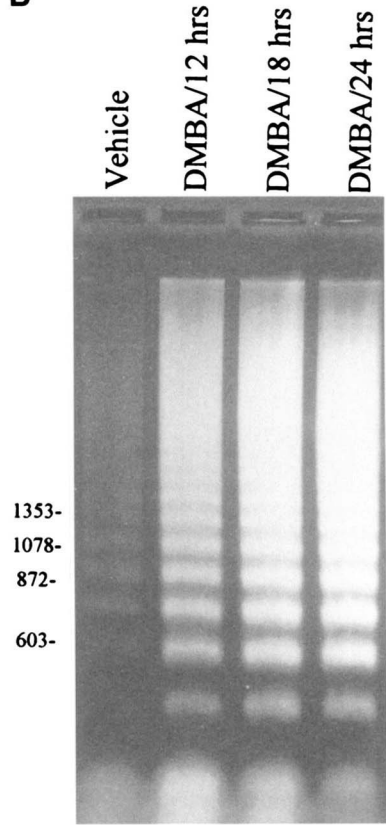
control cells (6%) exhibited the classic morphologic features of apoptotic cells, i.e., smaller (lower forward scatter) and somewhat more granular (increased side scatter). However, the percentage of apoptotic cells, as defined both by morphologic and DNA staining parameters, increased to approximately 35% following DMBA exposure. Cellular and nuclear condensation was readily observed by light microscopy. Similar results were obtained with BU-11 subclones and with primary preB cells from Whitlock/Witte (see Ref. 34) cultures (data not shown). Changes in PI staining and cell morphology correlated with DNA fragmentation characteristic of apoptosis (Fig. 2B); i.e., DNA extracted from BU-11 cells treated with $10^{-4}$ M DMBA
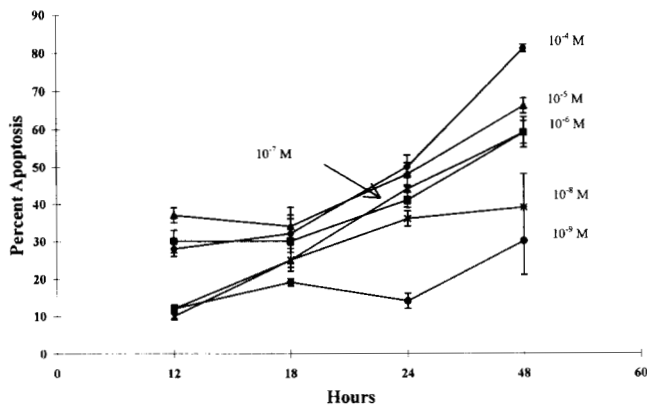
**FIGURE 3.** Kinetics and dose response of BU-11 cells to DMBA. Vehicle (0.1%) or DMBA ($10^{-4}$–$10^{-9}$ M) was added in duplicate to cultures of BU-11 cells maintained on BMS2 stromal cells. From 12 to 48 h later BU-11 cells were harvested and stained with PI, and the percentage of cells undergoing apoptosis was quantitated by flow cytometry. Data are pooled from a minimum of four experiments. Apoptosis in groups treated with vehicle from 12 to 24 h averaged 16 ± 1% with no significant differences at any time point. Apoptosis following DMBA exposure reached statistical significance relative to vehicle controls ($p < 0.01$) after 12 h of exposure to $10^{-4}$ to $10^{-6}$ M DMBA and after 24 h of exposure to $10^{-8}$ M DMBA.

Table I. *α-NF blocks DMBA-induced apoptosis in a PreB cell line (BU-11)* [a]

| Inhibitor | Vehicle | DMBA | | |
| --- | --- | --- | --- | --- |
| | | $10^{-4}$ M | $10^{-5}$ M | $10^{-6}$ M |
| | 15 ± 2 | 43 ± 2 | 37 ± 5 | 40 ± 4 |
| $10^{-6}$ M α-NF | 19 ± 2 | 33 ± 3* | 13 ± 1* | 17 ± 2* |

[a] Cultures of BU-11 cells maintained on BMS2 cell monolayers were treated for 24 h with vehicle or titered doses of DMBA ± α-NF. BU-11 cells were harvested from duplicate wells, stained with PI, and the percentage of cells undergoing apoptosis quantitated by flow cytometry. An asterisk indicates a significant inhibition of apoptosis relative to groups receiving the same dose of DMBA but no α-NF; $p < 0.05$. Data are pooled from three experiments.

for 12, 18, or 24 h and electrophoresed through 3.5% agarose gels exhibited a ladder pattern characteristic of digestion into oligonucleosomal fragments. No DNA digestion was evident in BMS2 cells (data not shown).

Kinetics and titration experiments demonstrated significant apoptosis in BU-11 cells as few as 12 h after addition of $10^{-4}$ to $10^{-6}$ M DMBA and at 24 h with as little as $10^{-8}$ M DMBA (Fig. 3, $p \leq 0.01$). These doses are comparable with those at which dexamethasone induces apoptosis in thymocytes (42). It should be noted that initial DMBA doses $\geq 10^{-5}$ M may be saturating, resulting in little or no differences in apoptosis induced at $10^{-4}$ to $10^5$ M DMBA over a 48-h period. Collectively, the data define one mechanism of AhR ligand immunotoxicity, i.e., induction of preB cell apoptosis in a relatively short period of time and at extremely low doses.

*DMBA-mediated apoptosis is blocked by α-NF, an AhR and cytochrome P-450 antagonist*

Activation of the AhR induces cytochrome P-450 (*CYP1A1* and *1A2*) gene transcription and enzyme activity (20). It is this AhR-regulated mono-oxygenase activity that initiates PAH metabolism. Several studies have demonstrated that both AhR translocation and cytochrome P-450 1A1 and 1A2 activity can be inhibited with flavones such as α-NF (20, 43). In particular, α-NF has been used extensively to confirm a role for the AhR or AhR-regulated enzymes in hydrocarbon immunosuppression (19, 20, 22, 44). To determine if the AhR and/or genes controlled by the AhR play a critical role in apoptosis induced by the known AhR ligand, DMBA, cultures of BU-11 and BMS2 cells were treated with vehicle or with $10^{-4}$ to $10^{-6}$ M DMBA with or without $10^{-6}$ M α-NF. While $10^{-6}$ M α-NF by itself had no effect on apoptosis, it significantly inhibited apoptosis induced with $10^{-4}$ M DMBA and completely blocked apoptosis induced with $10^{-5}$ or $10^{-6}$ M DMBA (Table I). The difference in the ability of $10^{-6}$ M α-NF to block apoptosis induced with $10^{-4}$ M as compared with $10^{-5}$ M DMBA suggests that DMBA saturation has not been reached at $10^{-5}$ M. In general, these results are consistent with the conclusion

that DMBA-induced apoptosis is mediated by the AhR and/or proteins regulated by the AhR (i.e., P-450 1A1 and 1A2).

*BMS2 but not BU-11 cells express high levels of AhR mRNA and protein*

Results described above support a role for the AhR in DMBA-induced apoptosis. However, they do not determine whether preB cells are the direct targets of DMBA activity or if B cell apoptosis is secondary to effects on stromal cells. To address this issue, the cellular distribution of AhR mRNA and protein was assessed.

In situ hybridization of BU-11/BMS2 cultures with an AhR antisense riboprobe revealed a strong AhR mRNA signal in BMS2 cells (Fig. 4, *A* and *B*, solid arrows). Significantly, no signal was detected in BU-11 cells (striped arrows). The specificity of in situ hybridization was confirmed by the lack of signal in cultures probed with an AhR sense riboprobe (Fig. 4C).

A more sensitive, AhR mRNA-specific RT-PCR was used to confirm these results. Indeed, no AhR mRNA was detected in BU-11 cells cultured for 7 days in rIL-7 (Fig. 5). However, high levels of AhR mRNA were present in BMS2 cells. Quantitation of AhR mRNA with a competitive AhR mimic indicated that the level of AhR mRNA in BMS2 was ~2,700 molecules/cell as compared with 10,600 molecules/Hepa-1 cell, a prototypic high AhR-expressing hepatoma line (1). Initial studies with BU-11 cells harvested directly from BMS2 monolayers and purified by one round of plate adherence demonstrated a very weak AhR band in some, but not all experiments (data not shown). Careful analysis correlated this band with low level (0.1 to 1%) BMS2 cell contamination. Therefore, it was necessary to pass rIL-7-maintained BU-11 cells several times to ensure the absence of BMS2 cells. Because of this protocol, it is formally possible that IL-7 growth affected AhR mRNA expression in BU-11 cells.

As expected from these results, AhR protein was detected in whole cell lysates of BMS2 but not in freshly isolated BU-11 cells (Fig. 6A). In close agreement with results obtained in the quantitative RT-PCR, density analysis of AhR bands in immunoblots indicated that BMS2 cells contain approximately one-fifth as much AhR protein as Hepa-1 cells. Similar results were obtained with polyclonal anti-AhR Abs (data not shown). Addition of DMBA, or a related AhR ligand, B[a]P, to BMS2 cultures induced translocation of the AhR to the nucleus (Fig. 6B). Nuclear translocation induced in Hepa-1 cells by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is shown for comparison. Densitometric analysis indicated a sixfold increase in BMS2 nuclear AhR induced with either PAH relative to vehicle controls. This increase in nuclear AhR could not have resulted from an overall increase in AhR in DMBA-treated cells since, as reported previously in other systems (10, 45), total levels of AhR decreased steadily during the 2.5 h following PAH exposure (Fig. 7). These results demonstrate that
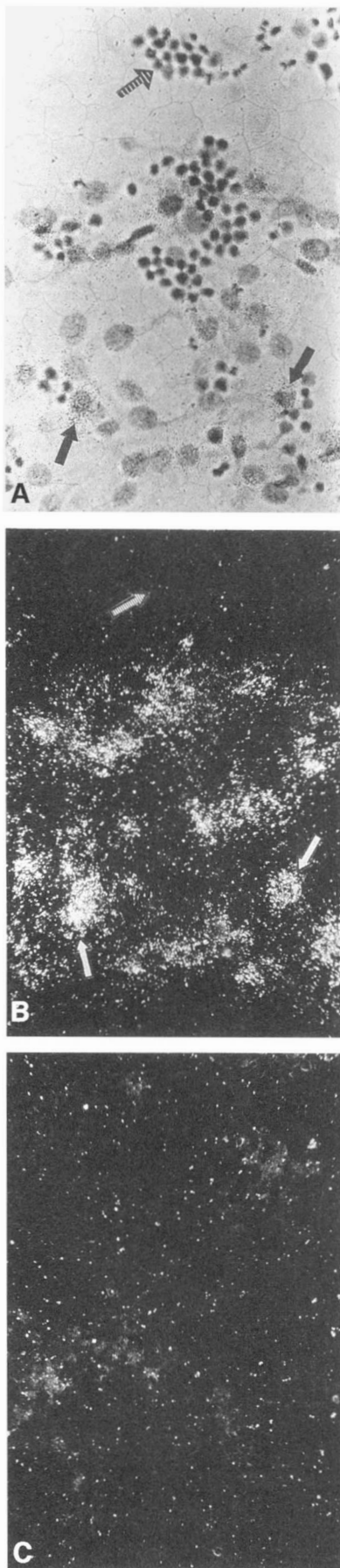
**FIGURE 4.** In situ hybridization for AhR mRNA. Cultures of BU-11 cells growing on BMS2 monolayers on glass slides were fixed with 4%
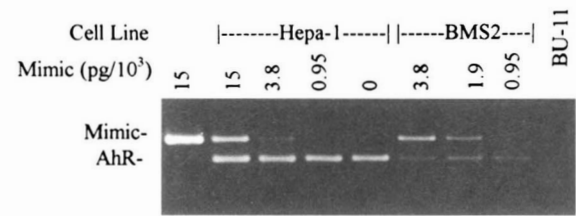


**FIGURE 5.** Quantitative RT-PCR for AhR mRNA in BMS2 and BU-11 cells. BU-11 cells were passed three to four times while maintained for 7 days in rIL-7 to insure the absence of contaminating BMS2 cells. RNA was extracted from Hepa-1, a liver hepatoma line (1), BMS2, or BU-11 cells, reverse transcribed and AhR mRNA amplified by PCR (35 cycles) in the presence of known amounts of AhR competitive mimic. Lanes in which the density of the AhR and AhR mimic bands are equal reveal the amount of AhR mRNA in the respective samples. BMS2 and Hepa-1 cells contain ~2,700 and 10,600 AhR mRNA molecules/cell, respectively.
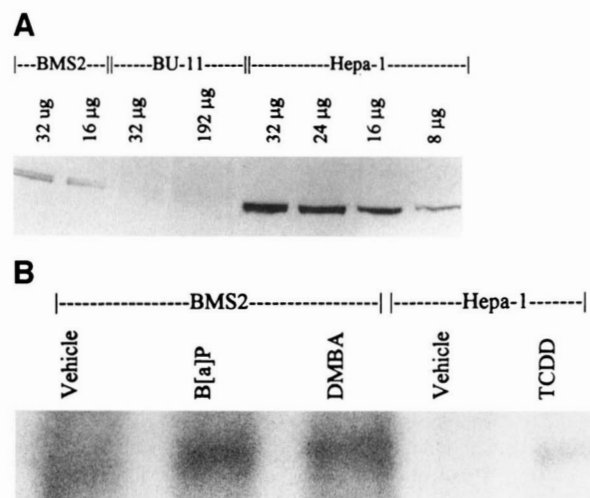


**FIGURE 6.** BMS2 but not BU-11 cells express functional AhR. Total cellular protein from untreated BMS2, freshly isolated BU-11 cells or Hepa-1 cells (*A*) or nuclear protein from BMS2 or Hepa-1 cells treated 60 min previously with vehicle-, $10^{-4}$ M B[a]P-, DMBA- or $10^{-8}$ M TCDD (*B*) was extracted, electrophoresed, transferred to nitrocellulose filters, and immunoblotted with monoclonal (RPT-1) anti-AhR Ab. For nuclear protein immunoblots, 15 $\mu$g of BMS2 nuclear protein and 5 $\mu$g of Hepa-1 nuclear protein were electrophoresed. The predominant 95- to 110-kDa AhR band is shown. BMS2 cells contain about one-fifth as much AhR protein as Hepa-1 cells. Data from a representative experiment (>10 total) are presented.

BMS2 cells contain the necessary elements (e.g., hsp90, Arnt) to facilitate AhR activation. Low levels of AhR were generally detected in nuclear preparations from vehicle or untreated cells, suggesting either low level contamination with cytosolic protein or constitutive activation of the AhR in BMS2 cells. A similar result has been reported with HeLa cells (46). Collectively, the data indicate that relatively high levels of functional AhR are expressed in BMS2 cells but little or no AhR is present in BU-11 cells.

To determine whether the failure to detect AhR in BU-11 cells reflects the level of AhR expressed in fresh bone marrow B cells,

paraformaldehyde, dehydrated, and hybridized for 18 h at 52°C with either AhR antisense (*A*, bright-field; *B*, dark-field) or sense (*C*) $^{35}$S-labeled riboprobes. Slides were washed and autoradiographs exposed for 90 days. Solid and striped arrows indicate BMS2 and BU-11 cells, respectively.
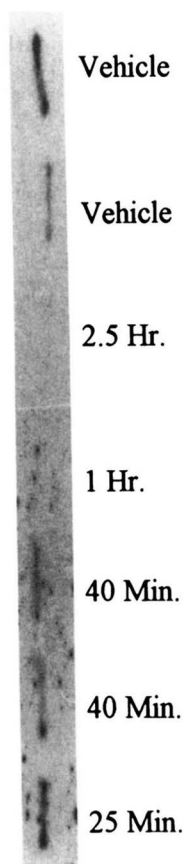
**FIGURE 7.** DMBA induces a loss of total AhR in BMS2 cells. BMS2 cells were treated with vehicle or $10^{-5}$ M DMBA for the periods indicated. Cells were harvested, washed, protein extracted, electrophoresed, transferred to nitrocellulose filters, and immunoblotted with monoclonal (RPT-1) anti-AhR Ab. The predominant 95- to 100-kDa AhR band is shown.
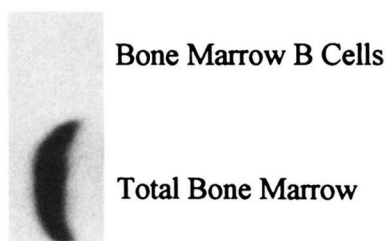


**FIGURE 8.** AhR is not detectable in primary bone marrow B cells. Bone marrow cells were expunged from the femurs of mice. Monocytes and stromal cells were depleted by adherence on culture dishes. Bone marrow B cells were then enriched on anti-CD45/B220 Ab-coated petri dishes. B cells were harvested, washed, protein extracted, electrophoresed, transferred to nitrocellulose filters, and immunoblotted with monoclonal (RPT-1) anti-AhR Ab. The predominant 95- to 100-kDa AhR band is shown.

CD45/B220$^+$ B cells were purified from bone marrow and assessed for AhR expression. Significant levels of AhR protein were detected in unfractionated bone marrow populations (15% CD45/B220$^+$). In contrast, no AhR was detected in highly enriched (>85% CD45/B220$^+$) bone marrow B cell populations (Fig. 8). Furthermore, AhR mRNA was not detected by RT-PCR in fresh bone marrow B cells purified by FACS (data not shown). These data support the validity of this model system.
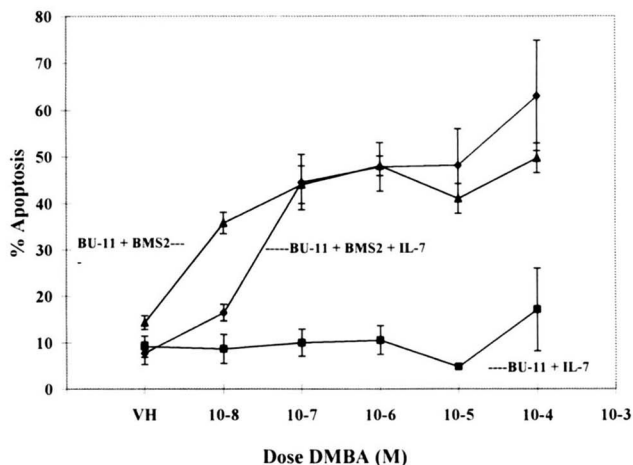


**FIGURE 9.** BMS2 cells are required for DMBA-induced preB cell apoptosis. Cultures of BU-11 cells maintained on BMS2 cells (BMS2 + BU-11), BU-11 cells maintained 10 to 14 days with rIL-7 in the absence of BMS2 cells (BU-11 + rIL-7), and BU-11 cells maintained on BMS2 cells with rIL-7 (BU-11 + BMS2 + rIL-7) were treated with vehicle, or $10^{-4}$ to $10^{-7}$ M DMBA. BU-11 cells were harvested 24 h later and apoptosis was quantitated with the PI/flow cytometry method.

*BMS2 cells are required for B cell apoptosis*

If DMBA-induced apoptosis is AhR dependent and only BMS2 cells express detectable AhR levels, it would be predicted that treatment of BU-11 cells with an AhR ligand in the absence of BMS2 cells would not result in apoptosis. To test this prediction, BU-11 cells were cultured for 10 to 14 days in rIL-7 during which time they were passed three to four times to ensure the absence of BMS2 cells. Cells were then treated with $10^{-4}$ to $10^{-8}$ M DMBA and apoptosis was quantitated 24 hr later. As predicted, BU-11 cells maintained in the absence of stromal cells were resistant to DMBA (Fig. 9, "BU-11 + IL-7"), even at $10^{-5}$ M DMBA, a dose 1000 times higher than the minimal dose required for apoptosis induction in the presence of BMS2 cells ("BU-11 + BMS2"). Furthermore, transfer of BU-11 cells maintained in rIL-7 to BMS2 stromal cell monolayers ("BU-11 + BMS2 + IL-7") reconstituted conditions for DMBA-induced apoptosis. These results rule out the possibility that BU-11 cells become DMBA resistant when cultured with rIL-7. In addition, since rIL-7 was present with BMS2, BU-11 cells, and DMBA, these experiments indicate that B cell apoptosis in the presence of BMS2 is not due to BMS2 loss of function, such as a reduction in IL-7 production, but rather to a gain of function, e.g., production of an as yet undefined factor. Finally, the requirement for AhR$^+$ stromal cells is consistent with the conclusion that the AhR is necessary for DMBA-induced apoptosis.

## Discussion

While the physiologic function of the AhR is not known, mounting evidence supports the hypothesis that, once activated, the AhR influences cell growth, function, and death. In the present report we support this hypothesis by demonstrating that, like some steroids, the common environmental pollutant and AhR ligand 7,12-dimethylbenz[a]anthracene induces apoptosis in immature lymphocytes. Several criteria were used to confirm DMBA induction of apoptosis: 1) a decrease in DNA staining with PI; 2) a change in cell morphology as assessed visually and by flow cytometry; and 3) digestion of DNA into oligonucleosomal fragments. From a biologic point of view, these results demonstrate that a functional, perhaps

active, AhR is expressed in primary lymphoid organs. Since apoptosis plays an important role in clonal selection and lymphocyte development (47), the results also have an important toxicologic implication, that exposure to low doses of common environmental pollutants may effect clonally nonspecific activation of a lymphocyte death program, compromising immune cell production and skewing lymphocyte repertoire development.

Data presented here support the hypothesis that PAH-induced preB cell apoptosis is dependent on a functional AhR. First, $\alpha$-NF completely blocks DMBA-induced apoptosis (Table I). $\alpha$-NF has been shown to be an AhR-competitive inhibitor (43, 48) and to block P-450 1A1 and 1A2 enzyme activity (20). It has been used by several groups to confirm a role for the AhR and/or AhR-regulated enzymes in immunosuppression (19, 20, 22, 44). Since P-450 1A1 and 1A2 levels are regulated by the AhR, inhibition of either AhR activation or P-450 activity by $\alpha$-NF would support AhR binding as a proximal event in PAH-induced apoptosis. Significantly, data presented elsewhere indicate that potent P-450 inhibitors have no effect on DMBA-induced apoptosis in BMS2 + BU-11 cell cultures and that P-450 1A1 or 1A2 activity is undetectable in BMS2 + BU-11 cultures (data not shown). These results argue against a role for P-450 enzymes in preB cell apoptosis and suggest direct AhR signaling for induction of the death signal. As would be required by this hypothesis, high levels of AhR are expressed in BMS2 stromal cells and can be rapidly activated by DMBA. These AhR$^+$ stromal cells are necessary for preB cell apoptosis as demonstrated by the failure of B cells grown in rIL-7 alone to die in the presence of DMBA at doses as high as $10^{-5}$ M (Fig. 9). (A low level of apoptosis at $10^{-4}$ M may have been due to AhR-independent signaling.) The failure of BU-11 cells grown in rIL-7 to respond to $10^{-5}$ to $10^{-8}$ M DMBA is not due to an IL-7 protective effect since BU-11 cells grown with rIL-7 and on BMS2 cells undergo DMBA-induced apoptosis. Finally, apoptosis cannot be induced with DMBA in BU-11 cells maintained on cell lines deficient in AhR signaling mechanisms (R. Near, R. A. Matulka, A. M. Shneider, K. K. Mann, S. U. Gogate, A. F. Trombino, and D. H. Sherr, manuscript in preparation).

Studies from other laboratories also support an immunomodulatory role for the AhR. For example, administration of PAH, including DMBA and B[a]P, or halogenated hydrocarbons suppresses immunity through AhR-dependent mechanisms (6, 23, 25, 49). Interestingly, some studies suggest that AhR ligands act directly on lymphocytes (50, 51). These studies may be distinguished from the present work in that they generally employed halogenated hydrocarbons rather than PAH. We have noted that TCDD, a halogenated hydrocarbon and high affinity AhR ligand, not only does not induce BU-11 cell apoptosis but blocks apoptosis induced with DMBA (data not shown). This result could reflect AhR blockade and/or delivery of an inhibitory signal. Therefore, it is possible that different biologic outcomes can be induced with different AhR ligands. Whether these outcomes reflect different AhR-binding affinities (52) remains to be determined. In addition, studies suggesting direct effects of AhR ligands on lymphocytes measured mature lymphocyte function while our work focused on the effects of PAH on immature, early preB cell survival. It is possible that mechanisms of AhR ligand immunosuppression reflect the differential expression or function of AhR in different cell subsets at distinct stages of lymphocyte development or activation. Consistent with differential expression of the AhR during lymphocyte development is the observation that significant levels of AhR can be detected in mature peripheral lymphocytes (53, 54) that respond to TCDD (51) but not in early preB cell lines (Figs. 4–6) or in primary bone marrow B cells (Fig. 8). In addition, we have observed only low levels of AhR in thymocytes purified by flow

cytometry (data not shown). Finally, it has recently been noted that AhR levels can be increased by activation of mature T lymphocytes through the TCR (32).

In apparent conflict with our results is the observation that DMBA can directly induce apoptosis in vitro in A20.1, a murine B cell lymphoma (55), albeit at significantly higher doses ($10^{-5}$ M) than those reported herein ($10^{-8}$ M). In our hands, A20.1 does not express AhR mRNA or protein. Therefore, it is likely that AhR-independent apoptosis signals can be induced directly in some lymphocytes at higher PAH doses. Indeed, we have shown that fluoranthene, a PAH that does not activate the AhR, induces apoptosis in AhR$^-$ T cell hybridomas, but only at doses significantly higher than those reported here with DMBA (56).

The requirement for bone marrow stromal cells for PAH-induced biologic activity is similar to results obtained with thymic cultures (30, 31) in which it was shown that the loss of immature thymocytes following exposure to TCDD is a function of thymic stromal cell changes. (These results also point out the possibility of different signals delivered by the AhR in different lymphoid tissues, i.e., in thymic vs bone marrow stroma). Furthermore, our studies are reminiscent of those demonstrating an indirect effect of xenobiotics on bone marrow stromal cells resulting in suppression of B lymphopoiesis (57).

None of these studies defined changes in the microenvironment that resulted in suppression of T or B lymphopoiesis. Since preB cells grow well in rIL-7, the ability to induce BU-11 preB cell apoptosis in the presence of stromal cells and rIL-7 (Fig. 7) demonstrates that DMBA-induced apoptosis does not result from loss of growth factor production but rather from a "gain of function." One candidate for the mediator of the apoptosis signal generated by stromal cells is TGF-$\beta$ since it has been shown to both oppose rIL-7-dependent preB cell growth in bone marrow cultures (58) and to be induced with AhR ligands (17). Experiments in progress are designed to determine if TGF-$\beta$, or other soluble factors play a role in DMBA-induced apoptosis. The possibility that membrane-bound Fas-Fas-ligand interactions are responsible for stromal cell-dependent BU-11 apoptosis has been considered. However, in contrast to our previous studies with activated T cells (47), Fas was not detectable on BU-11 cells and a soluble Fas-human Ig fusion protein failed to block PAH-induced preB cell apoptosis in primary preB cell (i.e., in Whitlock/Witte) cultures (data not shown). These data argue against a role for Fas in the present system. Regardless of the nature of the death signal delivered by stromal cells, preliminary data suggest that members of the BCL-2/BCL-X family play a role in apoptosis inasmuch as BCL-X$_L$ is up-regulated in BMS2 but not in BU-11 cells after DMBA exposure (data not shown).

Finally, our results build on the suggestion that the AhR plays a role in lymphocyte growth, development and/or function (29). Further experimentation with exogenous AhR ligands should provide important clues as to the physiologic function of this evolutionarily conserved (59) receptor/transcription factor.

## Acknowledgments

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