Regulation of PreB Cell Apoptosis by Aryl Hydrocarbon Receptor/Transcription Factor-Expressing Stromal/Adherent Cells (44410)

RICHARD I. NEAR,[1] RAYMOND A. MATULKA,* KOREN K. MANN,† SHAILA U. GOGATE,* ANTHONY F. TROMBINO,† AND DAVID H. SHERR*,†

Department of Environmental Health and Department of Pathology and Laboratory Medicine,† Boston University Schools of Medicine and Public Health, Boston, Massachusetts 02118*

Abstract. **Polycyclic aromatic hydrocarbons (PAH) are environmental chemicals that mediate immunosuppression. In long-term bone marrow B-cell lymphopoiesis models, PAH induce apoptosis in immature (preB) lymphocytes. Since the biologic function of PAH is often mediated by the aryl hydrocarbon receptor/transcription factor (AhR), the role of the AhR or AhR-regulated genes was assessed in preB cell apoptosis. Specifically, a bone marrow–derived preB cell line (BU-11) was cultured on monolayers of the AhR^+ bone marrow–derived stromal cell line BMS2, hepatoma sublines that express various levels of AhR activity (Hepa-1c1c7 and variants), AhR^+ thymic epithelial cells, and primary bone marrow stromal cells from wildtype or $AhR^{-/-}$ mice. Cultures were treated with one of two prototypic PAH, 7,12-dimethylbenz[*a*]anthracene (DMBA) or benz[*a*]pyrene (B[*a*]P), and the percentage of cells undergoing apoptosis measured. The data demonstrated that: 1) bone marrow- and hepatic-derived stromal/adherent cells support preB cell growth and regulate apoptosis induced by DMBA or B[*a*]P; 2) B[*a*]P is more effective than DMBA when preB cells are maintained on Hepa-1c1c7 monolayers than when maintained on BMS2 monolayers; 3) DMBA is more effective than B[*a*]P when preB cells are cultured on BMS2 monolayers; 4) α-naphthoflavone, an AhR antagonist and cytochrome P-450 inhibitor, blocks preB cell apoptosis in both BU-11/Hepa-1c1c7 and BU-11/BMS2 cultures; 5) although preB cells grow well in Hepa-1c1c7 or BMS2 supernatants, addition of PAH in the absence of hepatic- or bone marrow–derived adherent cells does not result in preB cell apoptosis; 6) preB cell apoptosis is dependent on AhR activity in adherent hepatic- or bone marrow–derived stromal cells; and 7) apoptosis is induced by DMBA when preB cells are maintained on primary bone marrow stromal cell monolayers from wildtype but not from $AhR^{-/-}$ mice. Collectively, the data indicated that AhR-regulated activities in the hematopoietic microenvironment influence the susceptibility of immature lymphocytes to low-dose PAH exposure.**

[P.S.E.B.M. 1999, Vol 221]

This work was supported by NIH Grant RO1-ES06086, Superfund Basic Research Grant #1P42ES 07381, ACS Grant IRG-97R, and a Veterans Administration Medical Research Division Center Grant to the Boston Environmental Hazard Center. K.K.M. was supported by an EPA STAR fellowship.

[1] To whom requests for reprints should be addressed at Department of Environmental Health, Boston University School of Medicine, 80 East Concord St. (S-105), Boston, MA 02118. E-mail: rocket1@bu.edu

Received September 18, 1998. [P.S.E.B.M. 1999, Vol 221]
Accepted March 4, 1999.

0037-9727/99/2213-0242$14.00/0

The aryl hydrocarbon receptor/transcription factor (AhR) is a 90-kDa heat shock protein– and XAP-2 (hepatitis B virus X-associated protein 2)–associated cytosolic protein that binds polycyclic aromatic (PAH) and halogenated aromatic hydrocarbons (HAH) (1, 2). Binding of these common environmental contaminants to the AhR is followed by receptor transport to the nucleus where the AhR associates with at least one cofactor, the aryl hydrocarbon nuclear translocator (ARNT) (3–6). Both the AhR and ARNT contain helix-loop-helix motifs and are members of the Per/ARNT/Sim (PAS) family of transcription factors

(5–9). The activated AhR-ARNT-ligand complex binds xenobiotic responsive elements (XRE) upstream from targeted genes and enhances their transcription (6, 10, 11). Increased transcription from a large number of genes is induced by the activation of the AhR upon ligand binding. A list of AhR-ligand inducible genes includes *CYP-1A1, 1A2,* and *1B1* (12, 13), proto-oncogenes c-*myc,* c-*erb*-A, c-*fos,* and c-*jun* (14–16), and genes encoding cytokines TGF-α, TGF-β, and interleukin-1β (17, 18).

In addition to inducing transformation of multiple cell types and influencing cell cycle (19, 20), AhR ligands such as 7,12-dimethylbenzanthracene (DMBA) and benzo[*a*]pyrene (B[*a*]P) frequently suppress both the cellular and humoral components of the immune response (21–34). HAH also generate profound immunosuppression manifest as thymic atrophy, inhibition of proliferation, and/or altered differentiation patterns in immature lymphocytes (23, 35–37). Indeed, the developing immune system appears to be particularly susceptible to AhR ligands (38–40). In some, but not all model systems, immunosuppression induced by PAH or HAH is dependent on AhR activation (22, 25, 28, 41).

Since stromal cells of the lymphopoietic microenvironment provide growth and differentiation signals to developing lymphocytes, we and others have suggested that the profound effects of AhR ligands on lymphocyte development could result from modulation of the cells that contribute to the lymphopoietic microenvironment (42–45). Previous studies demonstrating PAH-induced, preB cell apoptosis in long-term bone marrow cultures, a model system for B lymphopoiesis (46, 47), have been consistent with this hypothesis (42). In the present studies alternative microenvironments were provided for a bone marrow–derived preB line by a panel of stromal/adherent cells expressing various levels of AhR or by supernatants from stromal/adherent cell cultures. With these modifications it was possible to: 1) directly compare preB cell apoptosis induced by two prototypic PAH, B[*a*]P, and DMBA; 2) confirm a critical role for stromal/adherent cells in PAH-induced preB cell apoptosis; and 3) directly test the hypothesis that the AhR, and/or AhR-controlled genes, regulate PAH-induced preB cell apoptosis.

Materials and Methods

Cell Lines. Cell lines were maintained at 37°C in 10% CO_2 and, unless noted, in DMEM with 4.5 g/l glucose (BioWhittaker, Walkersville, MD) supplemented with 10% FCS (Gibco/BRL, Grand Island, NY), 2 m*M* L-glutamine (Gibco/BRL), 10 μg/ml gentamicin (Gibco/BRL), and 5×10^{-5} *M* 2-mercaptoethanol. PreB cell lines were obtained by harvesting stromal cell–adherent B cells from primary Whitlock-Witte cultures (43, 46) and transferring them to monolayers of a cloned bone marrow stromal cell line, BMS2 (47). Cells of one such line, termed BU-11, uniformly expressed the early preB cell marker CD43 and contained a rearranged immunoglobulin heavy chain gene (43). Since murine AhR polymorphisms affecting AhR expression and function have been reported, it should be noted that BU-11 and BMS2 cells were derived from C57BL/6 (AhR^{b-1}) and [B6 × D2]F1 (AhR^{b-1}/AhR^{d}) mice that express relatively high levels of high-affinity AhR (48).

BU-11 cells were grown on BMS2 monolayers and cells passed every 4 days at a 1:7 dilution onto BMS2 monolayers. Hepa-1c1c7 (Hepa-1; AhR^{b-1}) and its variant lines TAOc1BP^{r}c1 (Hepa-1TAO) and BP^{r}c1 (Hepa-1BP^{r}c1; Ref. 49) were obtained from Dr. J. P. Whitlock, Jr. (Stanford University), and cells passed every 4–7 days at a 1:10 dilution. The Hepa-1 mutant c2 (Hepa-1c2; Ref. 6) was obtained from Dr. O. Hankinson (UCLA) and cells passed every 4–7 days at 1:10. A fibroblast line derived from SIM mice and commonly used to support embryonic stem cells (STO), was obtained from Dr. M. Carroll (Harvard Medical School) and passed every 4–7 days at a 1:10 dilution. BU-11 cells were also grown long-term in Hepa-1 or BMS2 cell supernatant in the absence of an adherent monolayer by passage at a 1:5 dilution every 3–4 days. The murine TE427.1 thymic epithelial cell line (AhR^{b-1}) (50, 51) was obtained from Dr. S. Vukmanovic (NY University) and passed every 4–7 days at a 1:10 dilution. BU-11 growth was effectively supported by all the above adherent cell lines. Previous data showed that Il-7 supports BU-11 growth in the absence of supporting cells (43). Therefore, it is possible that Il-7 *per se,* secreted from the above adherent cell lines, provides the major growth support; however, we have not investigated this possibility.

$AhR^{+/-}$ mice (9) were the generous gift of Dr. C. Bradfield (University of Wisconsin Medical School). To generate primary bone marrow stromal cells from wildtype and $AhR^{-/-}$ mice, $AhR^{+/-}$ heterozygote breeding pairs were set up and offspring analyzed for AhR genotype by AhR-specific Southern analysis of tail DNA. Bone marrow was expunged from homozygous $AhR^{+/+}$ and $Ahr^{-/-}$ littermates, washed, and plated at a concentration of 10^6 cells/ml in DMEM supplemented with 20% FCS, l-glutamine, and 2-mercaptoethanol as described above. Culture dish–adherent monolayers were washed several times over the next 2-week period to remove hematopoietic cells. Cells were harvested by treatment with 0.25% trypsin and transferred to 75 cm^2 flasks for long-term culture.

Apoptosis Induction. Apoptosis experiments were performed in 24-well plates over a period of 5 days. Stromal/adherent cells ($7–10 \times 10^4$) were plated on Day 1 and 10^5 BU-11 cells were added on Day 2. On Day 3, PAH or vehicle (acetone, 0.1% final concentration) was added to triplicate wells for each condition. Forty-two hours later (optimized for signal to noise ratio in the P.I. staining assay) BU-11 cells from individual wells were collected by gently pipetting and harvesting 1.3 ml of suspended cells for DNA staining. Each well was assayed individually by flow cytometry and results of triplicate wells were averaged in each experiment. Triplicate wells were pooled for DNA extraction and gel electrophoresis. In experiments where α-naphthoflavone (α-NF; Sigma Chemical Co., St. Louis, MO)

was tested for inhibition of PAH-induced apoptosis, 10^{-6} *M* α-NF was added 3 hr before PAH.

Flow Cytometry Assay for Apoptosis. Propidium iodide staining and flow cytometry to quantitate apoptosis were performed as previously described (22). Briefly, cells were washed in cold PBS containing 1% FCS, pelleted, and resuspended in 0.5 ml hypotonic fluorochrome solution containing 50 μg/ml propidium iodide (P.I.) (Sigma Chemical Co., St. Louis, MO), 0.1% sodium citrate, and 0.1% Triton X-100 (Sigma). Cells were analyzed for P.I. fluorescence, forward light scatter, and side light scatter parameters with a Becton Dickenson (San Jose, CA) FACscan flow cytometer. Apoptotic cells were defined as those in which P.I. fluorescence was weaker than in the G_0/G_1 cell cycle peak.

DNA Fragmentation Gels. Approximately 10^6 cells washed once in cold RPMI were suspended in 4°C TEX (10 m*M* Tris pH 7.5/1 m*M* EDTA/0.2% Triton X-100) and incubated on ice for 7 min. Particulate matter was pelleted at 14,000 RPM. Sodium acetate (0.25 *M*) was added to the supernatants, and DNA was extracted with phenol/chloroform and precipitated with ethanol. The precipitate was dissolved in TE buffer (10 m*M* Tris pH 7.5/1 m*M* EDTA) and loading buffer (1/5 volume) containing TE, 1% SDS, 40% sucrose bromphenol blue, and 3 μg/ml RNaseA was added. DNA was then loaded into dry wells of a 1.5% agarose gel in Tris acetate buffer and electrophoresis begun. Once the samples completely entered the gel, the gel was totally submerged in TE buffer for electrophoresis.

Western Immunoblotting. Adherent stromal cells were harvested by a 3-min treatment with 0.25% trypsin (Gibco/BRL). Cells were washed twice in PBS, resuspended in lysing buffer (1% Triton X-100, 150 m*M* NaCl, 25 m*M* Tris/HCl, 1 μg/ml aprotenin, 10 μg/ml leupeptin, 1 m*M* EDTA, 50 m*M* NaF, 1 m*M* sodium orthovanadate, 1 m*M* PMSF) and centrifuged for 10 min at 15,000*g*. Protein concentrations in supernatants were measured with a bicinchoninic acid protein assay reagent kit (Pierce Chemical Co., Rockford, IL). Samples were analyzed by standard mini 10% SDS polyacrylamide gels. Proteins were transferred from gels to nitrocellulose filters (Bio-Rad, Hercules, CA) or PVDF paper (Millipore, Bedford, MA) at 150 volts for 1 hr. Transfer was monitored by staining membranes with Ponceau S (Sigma). Filters were blocked with TBST buffer (20 m*M* Tris, 0.5 *M* NaCl, 0.03% Tween 20, pH 7.5) containing 5% dry milk, washed twice for 5 min in TBST and incubated with monoclonal anti-AhR antibody Rpt1 (52) at a 1/10,000 dilution for 1 hr at room temperature. Filters were washed three times with TBST and incubated for 1 hr at room temperature with a 1:6,000 dilution of HRP-goat anti-mouse antibody (Sigma). Filters were washed twice and developed by enhanced chemiluminescence and autoradiography (DuPont NEN Research Products Co., Boston, MA).

RT-PCR for AhR mRNA. Adherent cells were grown on 10-cm dishes to near confluence and lysed directly with "RNAzol" (Leedo Medical Laboratories, Houston, TX); RNA was extracted as described by the manufacturer. BU-11 cells were harvested, centrifuged, and the pellet lysed with RNAzol. RNA samples (5-μg) were electrophoresed in 1.5% 10 m*M* phosphate agarose gels prior to RT-PCR to monitor RNA integrity.

RT-PCR was performed as described by the manufacturer (SuperScript Preamplification System; Gibco/BRL) using random hexamers to prime first strand cDNA synthesis. The $MgCl_2$ concentration was adjusted to 2.5 m*M* to maximize specific signal. The primers for amplifying AhR mRNA were: 5′-CTGGCAATGAATTTCCAAGGGAGG-3′ and 5′-CTTTCTCCAGTCTTAATCATGCG-3′. These primers were chosen to enclose the sequence that encodes the putative murine AhR ligand-binding domain (53). The random primed cDNA fragments were amplified with Taq for 35 cycles (94°C for 40 sec; 59°C for 50 sec; 72°C for 45 sec +2 sec/cycle). ARNT mRNA was generally amplified in the same reaction using the following ARNT-specific primers, 5′-CTTGGAGGCAGCAGATGGCTTTC-3′ and 5′-CCTTGATGTAGCCTGTGCAGTGG-3′. Amplified DNA (10 μl) was electrophoresed through 3% gels (3:1 NuSieve:LE agarose, FMC, Rockland, ME) and DNA visualized by ethidium bromide staining.

Results

Liver Parenchyma-Derived Hepa-1c1c7 Cells Support preB Cell Growth and PAH-Induced Apoptosis. Hepa-1c1c7 (Hepa-1) is a liver parenchyma-derived hepatoma that expresses high levels of AhR (10). A number of Hepa-1 variants exist that express variable levels of either AhR or the AhR dimerization partner ARNT (54). To investigate the mechanisms that contribute to PAH-induced B-cell apoptosis, particularly with regard to a role for the AhR, BU-11 preB cells were transferred from bone marrow stromal cell (BMS2) monolayers and adapted for growth on monolayers of adherent Hepa-1 cells. BU-11 cell growth rates and surface phenotype were identical whether BU-11 cells were maintained on BMS2 or Hepa-1 cells. BU-11 cells maintained on Hepa-1 monolayers underwent morphologic changes consistent with apoptosis (i.e., cellular and nuclear condensation) within 24 hr of exposure to 10^{-6} *M* B[*a*]P. DNA extracted from these BU-11 cells and electrophoresed through 1.5% agarose gels exhibited DNA-fragmentation ladders typical of apoptotic cells as early as 12 hr after exposure to 10^{-6} *M* B[*a*]P (Fig. 1A).

PAH-induced BU-11 apoptosis in the presence of Hepa-1 and BMS2 monolayers was quantitated by flow cytometry. BMS2/BU-11 co-cultures were challenged with 10^{-6} *M* DMBA, and Hepa-1/BU-11 co-cultures were challenged with 10^{-6} *M* B[*a*]P since these PAH generate the maximal levels of apoptosis in the respective culture systems (see below). Both Hepa-1 and BMS2 monolayers supported significant levels of preB cell apoptosis (Fig. 1B). While background levels of apoptosis in BU-11 cell populations were generally under 10%, apoptosis levels in the

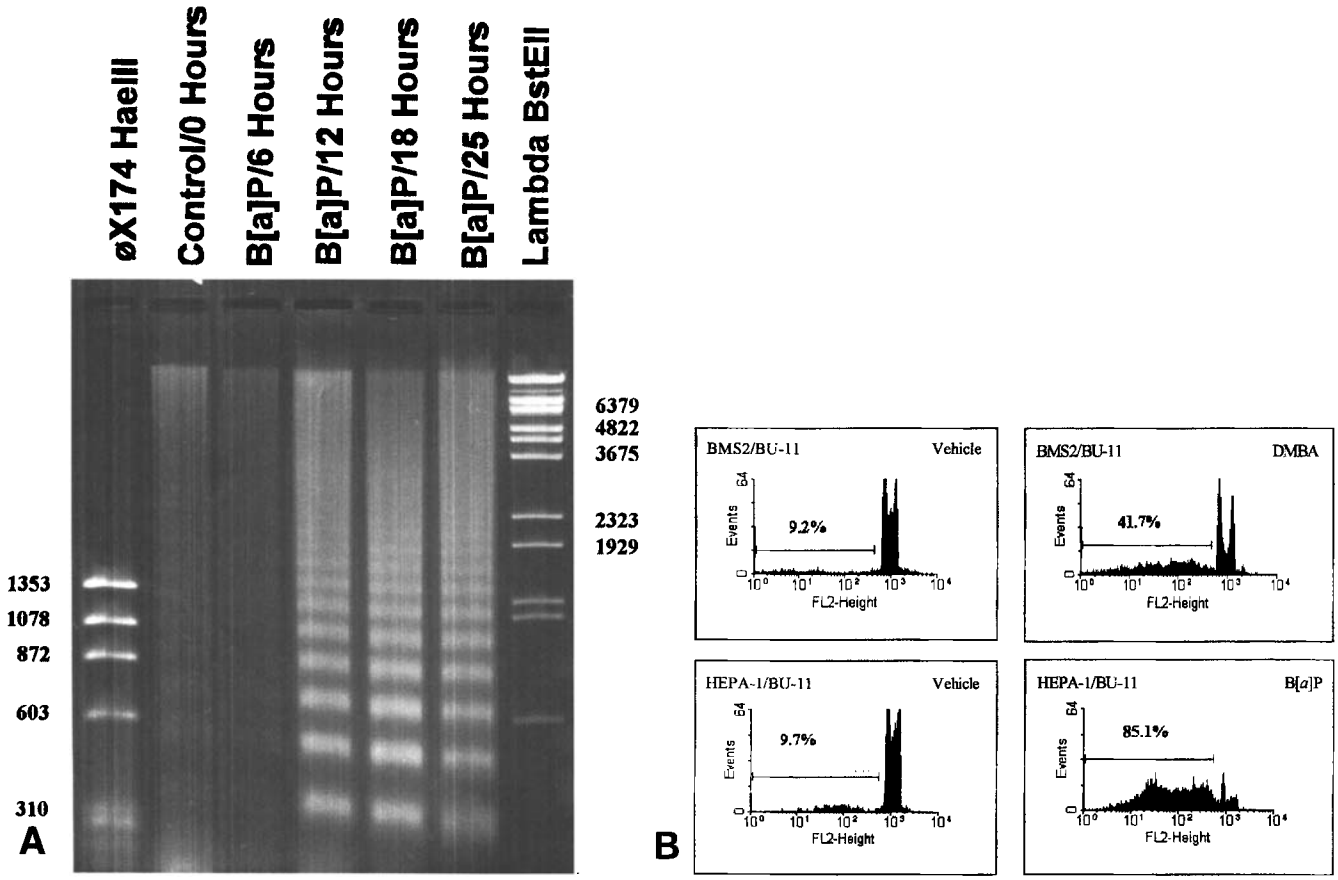

Figure 1. DMBA and B[*a*]P induce apoptosis in BU-11 cells. Vehicle, DMBA or B[*a*]P were added to BU-11 cells cultured on either Hepa-1 or BMS2 cell monolayers and assayed for apoptosis by DNA gel electrophoresis or flow cytometry. **(A)** Cultures of BU-11 cells growing on Hepa-1 cell monolayers were treated with vehicle ("control") or 10^{-6} *M* B[*a*]P and aliquots of approximately 10^6 BU-11 cells removed at various time points thereafter. DNA was extracted, electrophoresed through 1.5% agarose gels, and visualized with ethidium bromide. **(B)** Cultures of BU-11 cells growing on BMS2 or Hepa-1 cell monolayers were treated with 10^{-6} *M* DMBA or B[*a*]P respectively. Forty eight hours later BU-11 cells were harvested, stained with propidium iodide in hypotonic buffer, and analyzed for DNA staining by flow cytometry.

presence of B[*a*]P and Hepa-1 cell monolayers often reached 80%. The percentage of BU-11 cells undergoing apoptosis in BMS2 cultures treated with 10^{-6} *M* DMBA consistently reached 40%–50%. These results indicate that supporting cells other than those derived from the bone marrow microenvironment can provide an adequate environment for PAH-mediated preB cell apoptosis.

Adherent Cells Supporting preB Cell Growth Dictate Relative preB Cell Sensitivity to B[*a*]P and DMBA. Since the bone marrow and liver microenvironments may provide distinct signals to developing lymphocytes (55), the relative sensitivities of BU-11 preB cells to B[*a*]P and DMBA in the presence of BMS2 and Hepa-1 cell monolayers were assessed. Significant levels of apoptosis in BU-11 cell populations were observed at both DMBA or B[*a*]P doses tested, 10^{-6} and 10^{-5} *M*, regardless of which cell monolayer provided growth support ($P < 0.05$, Fig. 2). Higher levels of B[*a*]P-induced apoptosis were observed when BU-11 cells were grown on Hepa-1 monolayers (63 ± 14%, at 10^{-6} *M*) as compared to BU-11 cells grown on BMS2 monolayers (27 ± 4% at 10^{-6} *M*, $P < 0.01$). Conversely, higher levels of DMBA-induced apoptosis were reached when BU-11 cells were grown on BMS2 monolay-

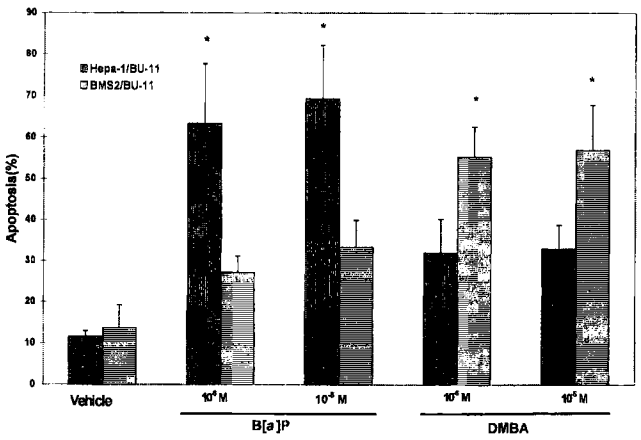

Figure 2. Stromal/adherent cells supporting preB cell growth dictate preB cell sensitivity to B[*a*]P and DMBA. Vehicle, DMBA or B[*a*]P were added to Hepa-1/BU-11 or BMS2/BU-11 cell cultures in triplicate wells. After 40 hr BU-11 cells were harvested, stained with propidium iodide in hypotonic buffer, and analyzed by flow cytometry. Data presented represent the average percentage of BU-11 cells undergoing apoptosis ± standard errors from three experiments. An asterisk indicates a significant difference in the percentage of apoptotic BU-11 cells between cultures treated with the same PAH at the same dose but with different supporting cell monolayers, $P < 0.05$. All PAH-treated cells exhibit apoptosis levels that are significantly above vehicle background, $P < 0.05$.

ers (55 ± 7% at 10^{-6} *M*) as compared with BU-11 cells supported by Hepa-1 cells (32 ± 8% at 10^{-6} *M*, $P < 0.02$). Lower doses of PAH, 10^{-7} *M* or lower, result in lower apoptotic levels; however, at these doses the differences of BU-11 apoptotic levels that result between B[*a*]P and DMBA exposure with BMS2 or Hepa-1 are statistically insignificant (not shown). This differential sensitivity to different PAHs was not due to adherent cell conditioning of BU-11 cells, since BU-11 cells cultured on Hepa-1 monolayers, transferred to BMS2 monolayers, and exposed to DMBA or B[*a*]P exhibited the same relative sensitivities as BU-11 cells constantly maintained on BMS2 monolayers (not shown). Therefore, the microenvironment provided by each supporting cell line regulates PAH efficacy as assayed by induction of preB cell apoptosis.

B[*a*]P- and DMBA-Induced Apoptosis are Inhibited by α-Naphthoflavone. Since DMBA and B[*a*]P are known to activate the AhR (6) it was predicted that the AhR, or genes controlled by the AhR (e.g., genes encoding cytochrome P-450s), would play a role in PAH-induced preB cell apoptosis. To test this hypothesis, cultures of BU-11 preB cells maintained on Hepa-1 monolayers were treated with DMBA or B[*a*]P in the presence or absence of α-NF, a known AhR antagonist and cytochrome P-4501A inhibitor (24, 26, 56). α-NF (10^{-6}) *M* had no affect on background levels of BU-11 cell apoptosis (Table I). In contrast, a significant percentage of BU-11 cells underwent apoptosis 42 hr following addition of either DMBA or B[*a*]P (Table I). DMBA-induced apoptosis was completely blocked and the majority of B[*a*]P-induced apoptosis (about 60% accounting for background apoptosis) was blocked by 10^{-6} *M* α-NF. Furthermore, B[*e*]P, a poorly metabolized and weak AhR ligand, did not induce BU-11 cell apoptosis (not shown). These results are consistent with the hypothesis that DMBA- and B[*a*]P-induced apoptosis of BU-11 cells in Hepa-1/BU-11 cultures is mediated at least in part by the AhR and/or AhR-controlled gene products.

BMS2 and Hepa-1 Cells, but not BU-11 preB Cells, Express AhR mRNA. If the AhR, or AhR-regulated gene products, contribute to PAH-induced preB cell apoptosis, then PAH exert their effects by directly interacting with AhR^+ preB cells and/or indirectly by affecting AhR^+-supporting cell monolayers. Previous work suggested the latter possibility since primary preB cells and BU-11 preB cells did not appear to express detectable levels of AhR mRNA or protein (43). To extend these findings and to address the possibility that culture of BU-11 cells on Hepa-1 cells induces AhR expression, BU-11, Hepa-1, and BMS2 cells were assayed for expression of AhR and ARNT mRNAs by RT-PCR. Consistent with previous reports, high levels of both AhR and ARNT mRNA were detected in Hepa-1 cells (Fig. 3). Similarly, significant levels of AhR and ARNT mRNA were detected in BMS2 cells. In contrast, no AhR mRNA was detected in BU-11 cells maintained either on BMS2 or Hepa-1 monolayers. Therefore, if the AhR or AhR-regulated genes influence PAH-induced preB cell apoptosis, then the most proximal effects of PAH exposure are likely to occur in supporting stromal/adherent cells that then indirectly effect preB cell apoptosis.

Interestingly, a significant amount of ARNT mRNA RT-PCR amplification product was observed from BU-11 mRNA (Fig. 3), suggesting independent regulation of the levels of mRNA expression of AhR and ARNT dimerization partners in immature lymphocytes. Furthermore, RT-PCR of BMS2 cell mRNA resulted in two different sized ARNT RT-PCR products (Fig. 3), whereas the RT-PCR of Hepa-1 cell mRNA showed only the expected, single 453-nucleotide product. This result could represent differential splicing of ARNT mRNA in BMS2 cells similar to that reported in human cell lines (6, 57).

Induction of preB Cell Apoptosis Requires AhR Complex-Competent Stromal Cells. Three approaches were taken to further test the hypothesis that preB cell apoptosis is effected through intermediary, AhR^+-supporting cells. In the first approach, the ability of PAH to induce apoptosis in BU-11 preB cells maintained in conditioned media in the absence of a supporting cell monolayer was evaluated. This approach was based on the demonstration that preB cells can be maintained for several weeks in IL-7. Indeed, BU-11 cells were easily grown in supernatant from either BMS2 or Hepa-1 cell cultures. When BU-11 cells were maintained in BMS2 or Hepa-1 culture supernatants and treated with vehicle, background levels of apoptosis averaged 11 ± 6% and 9 ± 2%, respectively, and did not significantly change following addition of 10^{-5}–10^{-7} *M* DMBA or B[*a*]P (Table II). Failure of BU-11 cells to undergo apoptosis was not likely due to PAH resistance acquired when grown in the absence of adherent supporting cells since BU-11 cell populations maintained in conditioned media, transferred back to Hepa-1 or BMS2 cell monolayers, and exposed to either DMBA or B[*a*]P, exhibited the same kinetics and magnitude of apoptosis as BU-11

Table I. α-Naphthoflavone Blocks DMBA and B[*a*]P-Induced Apoptosis of BU-11 PreB Cells in Hepa-1/BU-11 Co-Cultures

	Vehicle	α-NF	DMBA	DMBA + α-NF	B[*a*]P	B[*a*]P + α-NF
% Apoptosis	9.7 ± 1.8	6.1 ± 0.7	49.6 ± 1.8	8.4 ± 1.1*	82.7 ± 2.7	32.6 ± 2.0*

Note. BU-11 cells were cultured on Hepa-1 cell monolayers and treated in triplicate wells with vehicle, 10^{-6} *M* DMBA, 10^{-6} *M* B[*a*]P, 10^{-6} *M* α-NF or equimolar concentrations (10^{-6} *M*) of PAH + α-NF. BU-11 cells from triplicate wells were harvested after 42 hr, stained with propidium iodide, and the percentage of cells undergoing apoptosis in each well determined by flow cytometry. The three measurements of apoptosis for each group were averaged in each experiment. Data from 2–3 independent experiments are presented as the mean percentage apoptosis ± standard error. An asterisk denotes significant inhibition of apoptosis ($P < 0.02$).

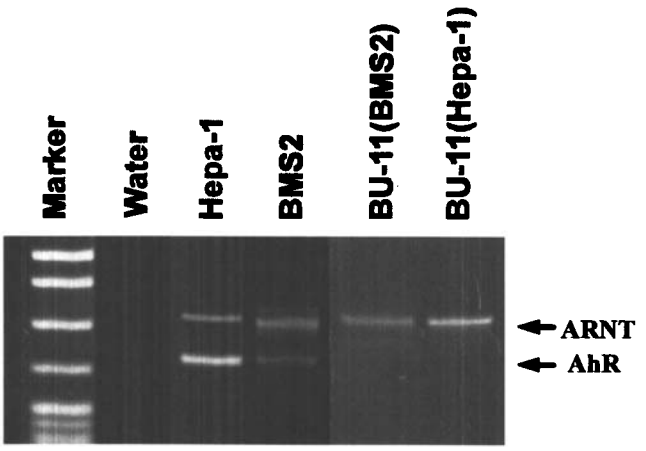

Figure 3. Relative expression of AhR and ARNT mRNAs in Hepa-1, BMS2, and Bu-11 cells. Culture dish–adherent, Hepa-1 and BMS2 cells were treated with 0.25% trypsin, harvested, washed, and whole-cell RNA extracted. BU-11 cells were grown in 0.04 μm filtered Hepa-1 or BMS2 cell supernatant to preclude contamination with stromal cells. RT-PCR was performed on 5 μg total RNA. AhR- and ARNT-specific primers were used simultaneously as described in "Materials and Methods" and PCR products examined by agarose gel electrophoresis. The BU-11 RT-PCR segment of the gel has different background than the rest of the gel since two gels were pasted together. Any background difference simply reflects more background ethidium bromide; relative comparison between AhR and ARNT bands are just as valid. AhR and ARNT mRNA RT-PCR products are 334 nt and 453 nt, respectively.

cell populations grown continuously on supporting cell monolayers (not shown). These results are consistent with a requirement for supporting cells for delivery of an apoptosis signal(s) to preB cells.

In the second approach, BU-11 cells were grown on seven different supporting stromal/adherent cell monolayers expressing various levels of AhR activity and tested for apoptosis in response to DMBA and B[*a*]P. Hepa-1TAO expresses AhR, which apparently is defective in PAH binding, and Hepa-1c2 cells produce little or no AhR (6, 49, 54). Hepa-1BP^{r}C1 is a Hepa-1 variant that expresses low levels of ARNT protein (49). STO is a mouse fibroblast line used to propagate embryonic stem cells (58), and TE427.1 is a thymic epithelial cell line that provides a microenvironment sufficient for thymocyte-positive selection (51). RT-PCR was performed to confirm or determine the levels of AhR and ARNT mRNAs in these supporting cell lines (Fig. 4). High levels of ARNT and AhR mRNAs were detected in Hepa-1 and in TE427.1 cells. In contrast, Hepa-1TAO, Hepa-1BP^{r}c1, Hepa-1c2, and STO cells were all defective in expression of AhR and/or ARNT mRNA.

Results obtained by AhR protein-specific immunoblotting were consistent with RT-PCR results in that high levels of AhR were detected in Hepa-1, Hepa-1BP^{r}c1, and TE427.1 cells. Little or no AhR was detected in STO, Hepa-1c2, or Hepa-1TAO cells (Fig. 5).

The levels of apoptosis induced by 10^{-6}–10^{-7} *M* DMBA or B[*a*]P in BU-11 cell populations maintained on the seven supporting cell monolayers are presented in Table II. Relative to vehicle controls, significant apoptosis in BU-11 cell populations was induced by the higher dose of either DMBA or B[*a*]P when BU-11 cells were supported by any of the AhR$^+$/ARNT$^+$ cell lines (i.e., BMS2, Hepa-1, or TE427). Significant apoptosis was induced in BU-11 cell populations from BMS2/BU-11 and TE427/BU-11 cultures exposed to 10^{-6}–10^{-7} *M* DMBA (up to 55% and 59%, respectively). In marked contrast, little or no apoptosis was induced by either PAH in BU-11 cell populations maintained on any of four AhR complex (i.e., AhR and/or ARNT)-defective cell lines (Hepa-1TAO, Hepa-1BP^{r}c1, Hepa-1c2, STO). The very low level of apoptosis observed in BU-11 cell populations from Hepa-1TAO/BU-11 cultures treated with B[*a*]P may reflect a low level of defective AhR reported to bind PAH, albeit weakly, and normal levels of ARNT in this Hepa-1 variant (Fig. 4; Refs. 49, 54). Similarly, the low level of apoptosis in Hepa-1BP^{r}c1 may reflect a low level of ARNT and normal AhR levels (Fig. 4; Refs. 49, 54). In general, the ability of DMBA and B[*a*]P to induce preB cell apoptosis correlated with the relative levels of both AhR and ARNT in the supporting stromal/adherent cell monolayers.

Finally, the ability of DMBA to induce BU-11 cell apoptosis in cultures of BU-11 cells and primary bone marrow stromal cells derived from either AhR$^{+/+}$ or AhR$^{-/-}$ mice was tested. Stromal cells from AhR$^{-/-}$ and AhR$^{+/+}$ mice were morphologically indistinguishable and were equally effective at supporting long-term BU-11 cell growth. BU-11 cells were transferred from BMS2 monolayers to monolayers of primary bone marrow stromal cells derived either from AhR$^{+/+}$ mice or from AhR$^{-/-}$ littermates. Vehicle or 10^{-5}–10^{-8} *M* DMBA was added 1 day later. Addition of as little as 10^{-8} *M* DMBA to AhR$^{+/+}$ stromal cell/BU-11 cultures significantly induced apoptosis in BU-11 cell populations within 24 hr (Fig. 6). Although there was a modest trend toward apoptosis at higher DMBA doses, statistically significant levels of apoptosis were not observed when BU-11 cells were supported by AhR$^{-/-}$ bone marrow stromal cells and cultures exposed to 10^{-5}–10^{-8} *M* DMBA. These results demonstrated that primary bone marrow stromal cells must express AhR in order for stromal cell–dependent preB cells to undergo significant apoptosis in response to DMBA exposure.

Discussion

The primary goal of the present studies was to test the hypothesis that the AhR and/or AhR-controlled gene products in the lymphoid microenvironment are required for PAH-induced preB cell apoptosis. These studies were motivated by numerous reports implicating a role for the AhR in suppression of mature lymphocyte responses (25, 28–30, 32, 41) and suggesting AhR-dependent modulation of lymphocyte development (59). To evaluate the specific requirement for AhR$^+$ stromal/adherent cells in DMBA and B[*a*]P-induced preB cell death, we modified a well-characterized model of B lymphopoiesis, in which preB cell growth is dependent on monolayers of adherent bone marrow stromal

Table II. AhR$^+$ Supporting Cells are Required for Optimal PAH-Induced PreB Cell Apoptosis

Supporting stromal/adherent cell line/supernatant	AhR/ARNT[a]	Vehicle	DMBA (*M*)		B[*a*]P (*M*)	
			10^{-6}	10^{-7}	10^{-6}	10^{-7}
BMS2 cells	+/+	13.6 ± 5.6	65.4 ± 10.4*	37.3 ± 11.7*	27.1 ± 4.1*	22.7 ± 9.7
BMS2 supernatant	NA	11.1 ± 6.3	17.6 ± 13.1	13.4 ± 7.5	14.1 ± 9.1	11.6 ± 7.1
Hepa-1 cells	++/++	11.5 ± 1.4	32.0 ± 8.2*	30.7 ± 8.0*	66.4 ± 16.1*	24.2 ± 2.7*
Hepa-1 supernatant	NA	9.3 ± 1.7	14.1 ± 1.4	8.9 ± 0.9	10.2 ± 0.7	11.2 ± 2.3
Hepa-1TAO cells	low/+	17.7 ± 2.2	19.7 ± 3.4	20.0 ± 3.9	24.9 ± 7.5	22.9 ± 3.2*
Hepa-1BP^{r}c1 cells	++/low	16.2 ± 4.4	16.8 ± 3.9	19.2 ± 7.3	25.7 ± 5.9*	23.8 ± 6.3
Hepa-1c2 cells	low/–	19.5 ± 3.1	17.2 ± 3.2	19.8 ± 2.0	21.2 ± 2.1	17.7 ± 0.9
STO cells	low/–	17.0 ± 4.2	20.8 ± 4.7	17.8 ± 5.7	20.8 ± 4.2	16.7 ± 6.2
TE427 cells	++/++	11.6 ± 5.7	58.8 ± 13.1*	46.5 ± 18.1*	41.0 ± 18.3*	14.1 ± 5.4

Note. BU-11 cells were maintained either in stromal/adherent (BMS2, Hepa-1) cell supernatants or monolayers of stromal/adherent cells as indicated. Vehicle, DMBA, or B[*a*]P were added to triplicate wells. After 42 hr, BU-11 cells from triplicate cells were harvested, and apoptosis in each well was quantitated by the propidium iodide/flow cytometry method. The three measurements of apoptosis for each group were averaged in each experiment. Data from 2–4 independent experiments are presented as the mean percentage apoptosis ± standard error. An asterisk indicates a significant increase in the percentage of apoptotic cells relative to vehicle controls, $P < 0.05$.

[a] Relative AhR and ARNT mRNA expression as assessed by RT-PCR (Fig. 4).

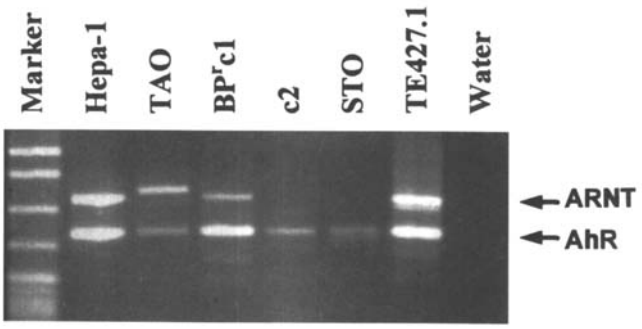

Figure 4. Relative expression of AhR and ARNT mRNA in Hepa-1 variants, STO fibroblasts, and TE427.1 thymic epithelial cells. Culture dish–adherent Hepa-1, Hepa-1TAO, Hepa-1BP^{r}C1, Hepa-1c2 cells, STO fibroblasts, and TE427.1 thymic epithelial cells were treated with 0.25% trypsin, harvested, washed, and whole-cell RNA extracted. AhR- and ARNT-specific RT-PCR was performed as described in Figure 3.

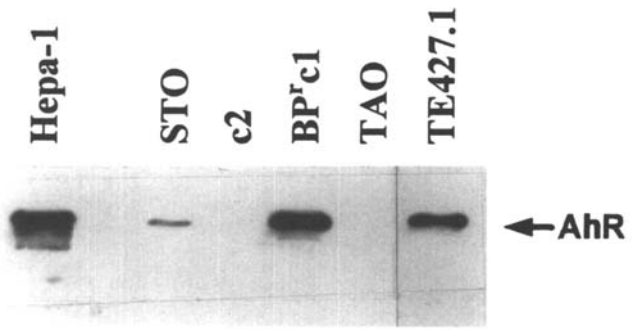

Figure 5. Relative expression of AhR protein in cells Hepa-1 variants, STO fibroblasts, and TE427.1 thymic epithelial cells. Culture dish–adherent Hepa-1, STO, Hepa-1c2, Hepa-1BP^{r}C1, Hepa-1TAO, and TE427.1 cells were treated with 0.25% trypsin, harvested, washed, and whole-cell protein extracted. Proteins (30 µg/lane) were electrophoresed through 10% SDS-polyacrylamide gels and transferred to nitrocellulose filters. AhR protein was detected with AhR-specific RPT-1 monoclonal antibody and HRP-goat anti-mouse immunoglobulin antibody and visualized by chemiluminescence.

cells. In the present system, parenchymal hepatic cells and their AhR complex variants, embryonic fibroblasts, thymic epithelial cells, or primary AhR$^-$ bone marrow stromal cells were substituted for AhR$^+$-cloned bone marrow stromal cells or AhR$^+$ primary bone marrow stromal cells. Conditioned media from stromal/adherent cells were also used to determine if PAH induce preB cell apoptosis in the absence of stromal/adherent accessory cells.

The results indicated that preB cells are not the immediate targets of PAH toxicity but rather that preB cell death is effected secondarily to changes occurring in supporting stromal or parenchymal accessory cells. When conditioned media from stromal/adherent cells were used instead of supporting monolayers, even relatively high doses of PAH did not induce preB cell apoptosis. Furthermore, the level of preB cell apoptosis directly correlated with the number of stromal/adherent cells present in culture (data not shown). By implicating nonlymphoid cells in the regulation of lymphocyte function, these results are reminiscent of reports implicating thymic epithelial cells as the primary targets of AhR ligand–mediated thymocyte immunosuppression (44, 45) and macrophages as a potential target of PAH-mediated suppression of B-cell responses (60). In a broader sense, nonlymphoid cells of the thymic or bone marrow microenvironment have been shown previously to regulate apoptosis in immature lymphocytes (61–63). The results presented here may be contrasted with those demonstrating that several other apoptosis-inducing agents (e.g., dexamethasone, prostaglandin E2, ceramide) induce immature lymphocyte apoptosis in the absence of accessory cells (64–66).

When stromal/adherent cells were co-cultured with preB (BU-11) cells at the time of PAH exposure, stromal/adherent cell lines expressing significant levels of AhR and ARNT (BMS2, Hepa-1, TE427) were capable of supporting maximal PAH-mediated preB cell apoptosis. In contrast, stromal/adherent cell lines expressing low or undetectable levels of AhR or ARNT (Hepa-1TAO, Hepa-1BP^{r}c1, STO) were either incapable or weakly capable of supporting apoptosis. Previous RT-PCR quantitation indicated 3–4-fold higher amounts of AhR-mRNA in Hepa-1 relative to BMS2 (43). It is possible that the higher levels of AhR in Hepa-1 may have a role in the induction of higher overall

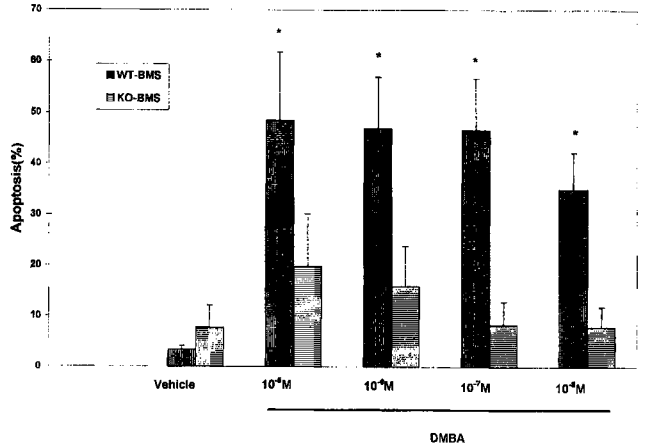

Figure 6. Primary bone marrow stromal cells from $AhR^{+/+}$ but not $AhR^{-/-}$ mice support optimal DMBA-induced preB cell apoptosis. Primary bone marrow stromal cells were harvested by trypsin treatment and transferred to 5-cm^2 wells at a concentration of 10^5 cells/ml. Twenty four hours later 5×10^4 BU-11 cells were added and allowed to adhere to stromal cell monolayers for 24 hr, after which vehicle or DMBA was added to duplicate cultures. Twenty four hours later BU-11 cells were harvested, stained with propidium iodide in hypotonic buffer, and analyzed for percentage of apoptotic cells. Data presented represent the average percentage of BU-11 cells undergoing apoptosis ± standard error from three experiments.

levels of BU-11 apoptosis supported by Hepa-1 versus BMS2 stroma *via* mechanisms that involve either direct AhR interactions or indirect transcriptional activation of genes affecting PAH metabolism such as CYP1A1 or CYP1B1 (73). PAH-induced preB cell apoptosis was the result of a "gain of function" and not a loss of stromal/adherent cell function *vis-a-vis:* 1) the growth or viability of the stromal/adherent cell lines tested was not overtly affected even by the highest PAH doses, and 2) since the presence of stromal/adherent cell-conditioned medium itself supports BU-11 cell growth, BU-11 preB cells in BMS2/BU-11 or Hepa-1/BU-11 co-cultures undergo PAH-induced apoptosis amid conditions that would otherwise support growth. These data strongly support the hypothesis that the AhR, and/or AhR-regulated gene products, are required for stromal/adherent cell generation of an apoptosis signal.

This conclusion was further strengthened by studies with stromal cells from $AhR^{+/+}$ and $AhR^{-/-}$ mice. DMBA at limiting concentrations (10^{-7}–10^{-8} *M*) induced apoptosis in co-cultures of wildtype preB cells and $AhR^{+/+}$ but not $AhR^{-/-}$ bone marrow stromal cells. At higher DMBA doses (10^{-6}–10^{-5} *M*), a statistically insignificant trend toward low levels of apoptosis in co-cultures of preB cells with $AhR^{-/-}$ bone marrow stromal cells was noted. This observation, and the failure of α-NF to block high-dose B[*a*]P-induced apoptosis completely (Table I), suggest a possible AhR-independent component to PAH-induced apoptosis. A similar AhR-independent component to an otherwise AhR-dependent suppression of mature lymphocyte responses has previously been reported (25). The potential to overcome AhR dependency at higher PAH doses may partially explain the sometimes conflicting reports on the role of the AhR in PAH-induced immunosuppression (25, 28–30, 32, 41).

There are multiple, nonmutually exclusive pathways through which the AhR could mediate stromal/adherent cell changes that lead to preB cell apoptosis. First, ample evidence provides support for direct intracellular signaling *via* an AhR-activated protein phosphorylation cascade (67). Second, the recent demonstration of AhR association with an immunophilin-like molecule (68, 69) suggests direct activation of intracellular signaling pathways analogous to those involved in cytokine regulation in lymphoid cells. Third, activated AhR may directly induce transcription of any one of a myriad of genes (70), the products of which could signal preB cell death. Finally, and arguably most likely, AhR-regulated P-450(s) could oxidize parent PAH resulting in production of reactive metabolites that alter cell function, possibly by inducing a state of oxidative stress and activating gene transcription factors such as NF-κB (71, 72). The dramatically reduced capacity of AhR complex–defective hepatic parenchymal cell lines and AhR^- bone marrow stromal cells of support apoptosis would then be attributed to their compromised ability to induce P-450 following PAH exposure (9).

Several predictions can be made based on the hypothesis that PAH metabolism is required for apoptosis. First, pharmacologic inhibitors of AhR activation and P-450 enzyme activity would be predicted to be potent inhibitors of PAH-induced apoptosis. Indeed, α-NF is both an AhR antagonist and P-450 inhibitor that inhibits PAH-induced apoptosis (Table I). Second, PAH that are metabolized differently in different stromal/adherent cells would be predicted to differ in their ability to induce apoptosis in preB cells co-cultured with these disparate stromal/adherent cells. Recent studies have demonstrated differential expression of cytochrome P-4501A1 and 1B1 in Hepa-1 and BMS2 cells (73). Since P-4501A1 and 1B1 are believed to be the most important P-450 isoforms for B[*a*]P and DMBA metabolism respectively, it would further be predicted that these PAH would exhibit different capacities to induce preB cell apoptosis in co-cultures of preB cells with Hepa-1 or with BMS2 cells. In fact, B[*a*]P is more potent in the Hepa-1/BU-11 system whereas DMBA is more potent in the BMS2/BU-11 system (Fig. 2). In addition, since B[*a*]P and DMBA have different AhR affinities (3), their reciprocal ability to induce apoptosis in the BMS2 and Hepa-1 cell systems argues against the possibility that AhR affinity alone determines immunotoxic potency. However, the role of P450 enzymes and corresponding metabolism will require further examination since preliminary experiments indicated that one general inhibitor 1-aminobenzotriazole (not shown) did not inhibit apoptosis whereas α-NF, which also inhibits certain P450s (and AhR), does block apoptosis (Table I and Ref. 43). Preliminary experiments indicate that stable metabolic products do not have a direct role in the induction of BU-11 apoptosis in this model, since supernatants taken from PAH-treated BMS2 or Hepa-1 cells and added to BU-11 cells grown alone, only in conditioned media from untreated BMS2 or Hepa-1 cells, do not induce BU-11 apop-

tosis. This, however, does not indicate whether or not metabolic products indirectly affect BU-11 cells by regulating the supporting stromal cells. Finally, if AhR ligand metabolism is critical to induction of the apoptosis signal, it would be predicted that TCDD, a high-affinity AhR ligand that is poorly metabolized, would be at best a weak inducer of apoptosis. In fact, TCDD does not induce apoptosis in either the BMS2/BU-11 or the Hepa-1/BU-11 system (data not shown). Clearly, additional studies are required to determine if PAH metabolism is necessary and/or sufficient for induction of the stromal/adherent cell–derived death signal.

The nature of the PAH-induced apoptosis signal derived from the stromal/adherent cells remains to be determined. However, experiments conducted thus far do not support a role for *Fas-Fas* ligand interactions, TGF, or TNF in PAH-induced preB cell apoptosis. Specifically: 1) *Fas* ligand is not induced in stromal cells, and preB cells do not express *Fas;* 2) DMBA does not induce TGF-β_1, β_2, β_3 or TNF-α mRNAs in bone marrow stromal cells; and 3) recombinant TGF-β_1 or TNF-α do not recapitulate preB cell apoptosis in stromal cell/preB cell cultures (data not shown).

Finally, the results presented here, as well as those previously reported, demonstrate that the developing immune system is particularly sensitive to AhR ligands. It is widely accepted that a critical phase in immune system development is deletion of autoantigen-reactive clones through apoptosis induction (74, 75). The ability of low PAH doses to activate the cell death program during lymphopoiesis in an antigen-independent fashion suggests the potential for these common environmental chemicals to skew the developing lymphocyte repertoire and to generally suppress production of mature lymphocytes. Either of these outcomes could significantly compromise the host's ability to respond to infectious agents and developing tumors.

The authors would like to thank Dr. P. Kincade for providing BMS2 cells, Dr. J. Whitlock for Hepa-1c1c7 cells and its variant lines TAOc1BP^{r}c1 and BP^{r}c1, Dr. O. Hankinson for the Hepa1c1c7 variant c2, Dr. M. Carroll for STO cells, Dr. S. Vukmanovic for TE417 cells, Dr. G. Perdew for RPT-1, anti-AhR antibody, and Dr. C. Bradfield for providing AhR$^{+/-}$ mice. The authors also thank J. Moniz for technical help.

1. Perdew GH. Association of the Ah receptor with the 90-kDa heat shock protein. J Biol Chem **263:**13802–13805. 1988.
2. Meyer BK, Pray-Grant MG, Vanden Heuvel JP, Perdew GH. Hepatitis B virus X-associated protein 2 is a subunit of the unliganded aryl hydrocarbon receptor core complex and exhibits transcriptional enhancer activity. Mol Cell Biol **18:**978–988, 1998.
3. Bigelow SW, Nebert DW. The Ah regulatory gene product, survey of nineteen polycyclic aromatic compounds' and fifteen benzo[*a*]pyrene metabolites' capacity to bind to the cytosolic receptor. Toxicol Lett **10:**109–118, 1982.
4. Cuthill S, Poellinger L, Gustafsson J-A. The receptor for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in the mouse hepatoma cell line Hepa-1c1c7. J Biol Chem **262:**3477–3481, 1987.
5. Dolwick KM, Swanson HI, Bradfield CA. *In vitro* analysis of Ah receptor domains involved in ligand-activated DNA recognition. Proc Natl Acad Sci USA **90:**8566–8570, 1993.
6. Hankinson O. The aryl hydrocarbon receptor complex. Annu Rev Pharmacol Toxicol **35:**307–340, 1995.
7. Ema M, Sogawa K, Watanabe N, Chujoh Y, Matsushita N, Gotoh O, Funae Y, Fujii-Kuriyama Y. cDNA cloning and structure of mouse putative Ah receptor. Biochem Biophys Res Commun **184:**246–253, 1992.
8. Probst MR, Fan CM, Tessier-Lavigne M, Hankinson O. Two murine homologs of the Drosophila single-minded protein that interact with the mouse aryl hydrocarbon receptor nuclear translocator protein. J Biol Chem **272:**4451–4457, 1997.
9. Schmidt JV, Su GH-T, Reddy JK, Simon MC, Bradfield CA. Characterization of a murine Ahr null allele: Involvement of the Ah receptor in hepatic growth and development. Proc Natl Acad Sci USA **93:**6731–6736, 1996.
10. Carrier F, Chang CY, Duh JL, Nebert DW, Puga A. Interaction of the regulatory domains of the murine *CyP1a1* gene with two DNA-binding proteins in addition to the Ah receptor and the Ah receptor nuclear translocator (ARNT). Biochem Pharmacol **48:**1767–1778, 1994.
11. Wen LP, Kociman N, Whitlock JP Jr. Dioxin-inducible, Ah receptor–dependent transcription *in vitro.* Proc Natl Acad Sci USA **85:**8545–8549, 1990.
12. Larsen M, Angus W, Brake P, Eltom S, Jefcoate C. CYP1B1 represents the major PAH-responsive P450 cytochrome constitutively expressed in normal primary HMEC. Fundam Appl Toxicol **36:**24–30, 1997.
13. Nebert DW, Gonzalez FJ. P450 genes: Structure, evolution, and regulation. Annu Rev Biochem **56:**945–993, 1987.
14. Bombick DW, Jankun J, Tullis K, Matsumura F. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin causes increases in expression of c-*erb*-A and levels of protein-tyrosine kinases in selected tissues of responsive mouse strains. Proc Natl Acad Sci USA **85:**4128–4132, 1988.
15. Puga A, Newbert DW, Carrier F. Dioxin induces expression of c-*fos* and c-*jun* proto-oncogenes and a large increase in transcription factor AP-1. DNA Cell Biol **11:**269–281, 1992.
16. Sadhu DN, Merchant M, Safe SH, Ramos KS. Modulation of proto-oncogene expression in rat aortic smooth muscle cells by benzo[*a*]-pyrene. Arch Biochem Biophys **300:**124–131, 1993.
17. Sutter TR, Guzman K, Dold KM, Greenlee WF. Targets for dioxin: Genes for plasminogen activator inhibitor-2 and interleukin-1β. Science **254:**415–418, 1991.
18. Gaido KW, Maness SC. Regulation of gene expression and acceleration of differentiation in human keratinocytes by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Toxicol Appl Pharmacol **127:**199–208, 1994.
19. Ma X, Babish JG. Acute 2,3,7,8-tetrachlorodibenzo-*p*-dioxin exposure results in enhanced tyrosylphosphorylation and expression of murine hepatic cyclin dependent kinases. Biochem Biophys Res Commun **197:**1070–1077, 1993.
20. Ma Q, Whitlock JP Jr. The aromatic hydrocarbon receptor modulates the Hepa-1c1c7 cell cycle differentiated state independently of dioxin. Mol Cell Biol **16:**2144–2150, 1996.
21. Clark GC, Blank J, Germolec D, Luster MI. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin stimulation of tyrosine phosphorylation in B lymphocytes: Potential role in immunosuppression. Mol Pharmacol **39:**495–501, 1991.
22. Hardin JA, Hinoshita F, Sherr DH. Mechanisms by which benzo[*a*]-pyrene, an environmental carcinogen, suppresses B cell lymphopoiesis. Toxicol Appl Pharmacol **117:**155–164, 1992.
23. Holsapple MP, Morris DL, Wood SC, Snyder NK. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin-induced changes in immunocompetence: Possible mechanisms. Annu Rev Pharmacol Toxicol **31:**73–100, 1991.
24. Kawabata TT, White KL. Suppression of the *in vitro* humoral immune response of mouse splenocytes by benzo[*a*]pyrene metabolites and inhibition of benzo[*a*]pyrene-induced immunosuppression by α-naphthaflavone. Cancer Res **47:**2317–2322, 1987.
25. Kerkvliet NI, Steppan LB, Brauner JA, Deyo HA, Henderson MC, Tomar RS, Buhler DR. Influence of the Ah locus on the humoral

immunotoxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin: Evidence for Ah-receptor-dependent and Ah-receptor-independent mechanisms of immunosuppression. Toxicol Appl Pharmacol **105:**26–36, 1990.

26. Ladics GS, Kawabata TT, White KL Jr. Suppression of the *in vitro* humoral immune response of mouse splenocytes by 7,12-dimethylbenz[*a*]anthracene metabolites and inhibition of immunosuppression by α-naphthoflavone. Toxicol Appl Pharmacol **110:**31–44, 1991.
27. Morris DL, Snyder NK, Gokani V, Blair RE, Holsapple MP. Enhanced suppression of humoral immunity in DBA/2 mice following subchronic exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Toxicol Appl Pharmacol **112:**128–132, 1992.
28. Morris DL, Jeong HG, Stevens WD, Chun YJ, Karras JG, Holsapple MP. Serum modulation of the effects of TCDD on the *in vitro* antibody response and on enzyme induction in primary hepatocytes. Immunopharmacol **27:**93–105, 1994.
29. White KL Jr., Lysy HH, Holsapple MP. Immunosuppression by polycyclic aromatic hydrocarbons: A structure-activity relationship in B6C3F1 and DBA/2 mice. Immunopharmacol **9:**155–164, 1985.
30. Wojdani A, Attarzadeh M, Wode-Tsadik G, Alfred LJ. Immunocytotoxicity effects of polycyclic aromatic hydrocarbons on mouse lymphocytes. Toxicology **31:**181–189, 1984.
31. Burchiel SW, Hadley WM, Barton SL, Fincher RH, Lauder LD, Dean JH. Persistent suppression of humoral immunity produced by 7,12-dimethylbenz[*a*]anthracene (DMBA) in B6C3F1 mice: Correlation with changes inn spleen cell surface markers detected by flow cytometry. Int J Immunopharmacol **10:**369–376, 1988.
32. Thurmond LM, House RV, Lauer LD, Dean JH. Suppression of splenic lymphocyte function by 7,12-dimethylbenz[*a*]anthracene in murine lymphoid cells. Toxicol Appl Pharmacol **93:**369–377, 1988.
33. Ward EC, Murray MJ, Lauder LD, House RV, Dean JH. Persistent suppression of humoral and cells mediated immunity in mice following exposure to the polycyclic aromatic hydrocarbon, 7,12-dimethylbenz[*a*]anthracene. Int J Immunopharmacol **8:**13–22, 1986.
34. Dean JH, Luster MI, Boorman GA, Lauer LD, Luebke RW, Lawson LD. Immune suppression following exposure of mice to the carcinogen benzo[*a*]pyrene but not the noncarcinogenic benzo[*e*]pyrene. Clin Exp Immunol **52:**199, 1983.
35. Poland A, Knutson JC. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin and related halogenated aromatic hydrocarbons: Examination of the mechanism of toxicity. Annu Rev Pharmacol Toxicol **22:**517–554, 1982.
36. Silverstone AE, Frazier DE Jr, Fiore NC, Soults JA, Gasciewicz TA. Dexamethasone, β-estradiol, and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin elicit thymic atrophy through different cellular targets. Toxicol Appl Pharmacol **126:**248, 1994.
37. Blaylock BL, Holliday SD, Comment CE, Heindel JJ, Luster MI. Exposure to tetrachlorodibenzo-*p*-dioxin (TCDD) alters fetal thymocyte maturation. Toxicol Appl Pharmacol **112:**207–213, 1992.
38. Holladay SD, Smith B. Alterations in murine fetal thymus and liver hematopoietic cell populations following developmental exposure to 7,12-dimethylbenz[*a*]anthracene. Exp Res **68:**106–113, 1995.
39. Holladay SD, Smith BJ. Fetal hematopoietic alterations after maternal exposure to benzo[*a*]pyrene: A cytometric evaluation. J Toxicol Environ Health **42:**259–273, 1994.
40. Fine JS, Silverstone AE, Gasiewicz TA. Impairment of prothymocyte activity by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. J Immunol **144:**1169–1176, 1990.
41. Harper N, Steinberg M, Thomsen J, Safe S. Halogenated aromatic hydrocarbon-induced suppression of the plaque-forming cell response in B6C3F1 splenocytes cultured with allogeneic mouse serum: Ah receptor structure activity relationships. Toxicol **99:**199–206, 1995.
42. Yamaguchi K, Matulka RA, Shneider A, Toselli P, Tombino AF, Yang S, Hafer LJ, Mann KK, Tao X-J, Tilly JL, Near R, Sherr DH. Induction of preB cell apoptosis by 7,12 dimethylbenz[*a*]anthracene in long-term bone marrow cultures. Toxicol Appl Pharmacol **147:**190–203, 1997.
43. Yamaguchi K, Near RI, Matulka RA, Shneider A, Toselli P, Trombino AF, Sherr DH. Activation of the aryl hydrocarbon receptor/transcription factor and bone marrow stromal cell–dependent preB cell apoptosis. J Immunol **158:**2165–2173, 1997.
44. Greenlee WF, Dold KM, Irons RD, Osborne R. Evidence for direct action of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on thymic epithelium. Toxicol Appl Pharmacol **79:**112–120, 1985.
45. Kremer J, Gleichmann E, Esser C. Thymic stroma exposed to aryl hydrocarbon receptor–binding xenobiotics fails to support proliferation of early thymocytes but induces differentiation. J Immunol **153:**2778–2786, 1994.
46. Whitlock CA, Robertson D, Witte ON. Murine B-cell lymphopoiesis in long-term culture. J Immunol Methods **67:**353–369, 1984.
47. Pietrangeli CE, Hayashi S-I, Kincade PW. Stromal cell lines which support lymphocyte growth: Characterization, sensitivity to radiation and responsiveness to growth factors. Eur J Immunol **18:**863–872, 1988.
48. Chang C, Smith DR, Prasad VS, Sidman CL, Nebert DW, Puga A. Ten nucleotide differences, five of which cause amino acid changes, are associated with the Ah receptor locus polymorphism of C57BL/6 and DBA/2 mice. Pharmacogenetics **3:**312–321, 1993.
49. Miller AG, Israel D, Whitlock JP Jr. Biochemical and genetic analysis of variant mouse hepatoma cell defective in the induction of benzo[*a*]-pyrene-metabolizing enzyme activity. J Biol Chem **258:**3523–3527, 1983.
50. Faas SJ, Rothstein JL, Kreider BL, Rovera G, Knowles BB. Phenotypically diverse mouse thymic stromal cell lines which induce proliferation and differentiation of hemotopoietic cells. Eur J Immunol **23:**1201–1214, 1993.
51. Vukmanovic S, Stells G, King PD, Dyall R, Hogquist KA, Harty JT, Nikolic-Zugic J, Bevan MJ. A positive selecting thymic epithelial cell line lacks costimulatory activity. J Immunol **152:**3814–3823, 1994.
52. Singh SS, Perdew GW. Alterations in the AhR level after staurosporine treatment. Arch Biochem Biophys **305:**170–175, 1993.
53. Burbach KM, Poland A, Bradfield CA. Cloning of the Ah-receptor cDNA reveals a distinctive ligand-activated transcription factor. Proc Natl Acad Sci USA **89:**8185–8189, 1992.
54. Israel D, Whitlock JP Jr. Induction of mRNA for cytochrome P1-450 in wild type and variant mouse hepatoma lines. J Biol Chem **258:**10390–10394, 1983.
55. Hardy RR, Li YS, Hayakawa K. Distinctive developmental origins and specificities of the $CD5^+$ B-cell subset. Seminars Immunol **8:**37, 1996.
56. Gasiewicz TA, Rucci G. α-Naphthoflavone acts as an antagonist of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin by forming an inactive complex with the Ah receptor. Mol Pharmacol **40:**607–612, 1991.
57. Hoffman EC, Reyes H, Chu F, Sander F, Conley LH, Brooks BA, Hankinson O. Cloning of a factor required for activity of the Ah (Dioxin) receptor. Science **252:**954–958, 1991.
58. Hogan B, Beddington R, Constantini F, Lacy E. Manipulating the mouse embryo: A laboratory manual (2nd ed). Plainview, NY: Cold Spring Harbor Press, 1994.
59. Fernandez-Salguero P, Pineau T, Hilbert DM, McPhail T, Lee SS, Kimura S, Nebert DW, Rudikoff S, Ward JM, Gonzalez FJ. Immune system impairment and hepatic fibrosis in mice lacking the dioxin-binding Ah receptor. Science **268:**722–726, 1995.
60. Ladics GS, Kawabata TT, Munson AE, White KL Jr. Evaluation of murine splenic cell type metabolism of benzo[*a*]pyrene and functionality *in vitro* following repeated *in vivo* exposure to benzo[*a*]pyrene. Toxicol Appl Pharmacol **116:**258–266, 1992.
61. Ashwell JD, King LB, Vacchio MS. Cross-talk between the T-cell antigen receptor and the glucocorticoid receptor regulates thymocyte development. Stem Cells **14:**490–500, 1996.
62. Fehsel K, Kroncke KD, Meyer ML, Huber H, Wahn V, Kolb-Bachofen V. Nitric oxide induces apoptosis in mouse thymocytes. J Immunol **155:**2858–2865, 1995.
63. Lerner A, Clayton LK, Mizoguchi E, Ghendler Y, Ewijk W, Koyasu S, Bhan AK, Reinherz EL. Cross-linking of T-cell receptors on double-positive thymocytes induces a cytokine-mediated stromal activation process linked to cell death. EMBO J **15:**5876–5887, 1996.

64. Brown DM, Warner GL, Alex-Martinez JE, Scott DW, Phipps RP. Prostaglandin E_2 induces apoptosis in immature normal and malignant B lymphocytes. Immunol Immunopathol **63:**221, 1992.
65. Zhi-Jun Y, Sriranganathan N, Vaught T, Arastu SK, Ahmed SA. A dye-based lymphocyte proliferation assay that permits multiple immunological analyses: mRNA, cytogenetic, apoptosis, and immunophenotyping studies. J Immunol Meth **210:**25–39, 1997.
66. Wiesner DA, Kilkus JP, Gottschalk AR, Quintans J, Dawson G. Anti-immunoglobulin-induced apoptosis in WEHI 231 cells involves the slow formation of ceramide from sphingomyelin and is blocked by BCL-X_L. J Biol Chem **272:**9868–9876, 1997.
67. Enan E, Matsumura F. Identification of c-*Src* as the integral component of the cytosolic Ah receptor complex, transducing the signal of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) through the protein phosphorylation pathway. Biochem Pharmacol **52:**1599–1612, 1996.
68. Carver LA, Bradfield CA. Ligand-dependent interaction of the aryl hydrocarbon receptor with a novel immunophilin homolog *in vivo*. J Biol Chem **272:**11452–11456, 1997.
69. Ma Q, Whitlock JP Jr. A novel cytoplasmic protein that interacts with the Ah receptor, contains tetratricopeptide repeat motifs, and augments the transcriptional response to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. J Biol Chem **272:**8878–8884, 1997.
70. Lai Z-W, Pineau T, Esser C. Identification of dioxin-responsive elements (DREs) in the 5′ regions of putative dioxin-inducible genes. Chem Biol Interact **100:**97–112, 1996.
71. Baldwin AS Jr. The NF-κB and IκB proteins: New discoveries and insights. Annu Rev Immunol **14:**649–681, 1996.
72. Baeuerle PA, Henkel T. Function and activation of NF-κB in the immune system. Annu Rev Immunol **12:**141–179, 1994.
73. Heidel SM, Czuprynski CJ, Jefcoate CR. Bone marrow stromal cells constitutively express high levels of cytochrome P4501B1 that metabolize 7,12-dimethylbenz[a]anthracene. Mol Pharmacol **54:**1000–1006, 1998.
74. Cui H, Ju S-T, Sherr DH. Functional expression of *Fas* (CD95) protein in autoimmune *lpr* mice. Cell Immunol **174:**35–41, 1996.
75. Ju S-T, Panka DJ, Cui H, Ettinger R, El-Khatib M, Sherr DH, Stanger BZ, Marshak-Rothstein A. The molecular mechanisms of programmed cell death following T-cell activation requires *Fas/Fas*L interactions. Nature **373:**444, 1995.