Polycyclic' Aromatic Hydrocarbon Induced Intracellular Signaling and Lymphocyte Apoptosis

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Ph.D. Thesis

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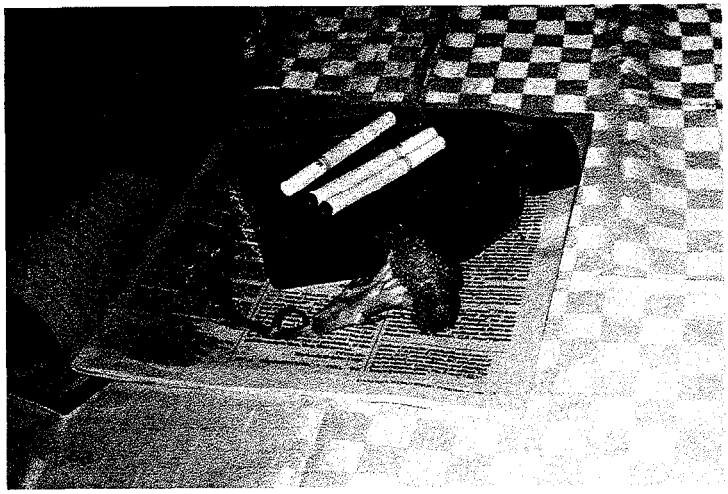

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USA

ABSTRACT.

The aryl hydrocarbon (dioxin) receptor (AhR) is a transcription factor possessing high affinity to potent environmental pollutants, polycyclic aromatic hydrocarbons (PAH) and related halogenated hydrocarbons (e.g. dioxins). Numerous research attribute toxicity of these compounds to the receptor mediated events.

It raises the question," Which additional transcription factors does the AhR interact with?" In the present work, the hypothesis was postulated and tested that the AhR ligated with PAH activates cytoplasmic NF-κB. This suggests a number of new toxic outcomes for PAH exposures, and offers novel mechanistic explanations for the toxicity of the known compounds.

Another unanswered question of the AhR biochemistry is," Which factors do control the AhR expression and activity?" Using fibroblast model, the role of a cell cycle in maintaining the AhR level was evaluated. The results of this research indicate that the AhR is controlled by the cell progression through the cell cycle. This may imply differential cellular sensitivity to the toxins, and a role for the AhR in cell growth/differentiation.

Previous PAH immunotoxicity research did not adequately address effects on immature lymphocytes. Our experiments on preB lymphocytes supported by stromal cells suggest that apoptosis is one of the mechanisms for PAH immunosuppression. It could be either due to direct effect of the PAH on the B cells, via stromal cell signaling.

Ubiquitous PAH-like toxin, fluoranthene, was tested for it's ability to initiate apoptosis in T cell hybridomas. Our data demonstrate that PAH may induce apoptosis and immunotoxicity in T cell branch of immune system. The mechanism of this process seems to be the AhR independent, and mediated by Ca^{2+}.

CONTENT

PREFACE.

The present study was carried out at the Department of Environmental Health, School of Public Health in the School of Medicine, Boston University, during the years 1993-1997.

I want to express my deepest gratitude to Professor David H. Sherr, Ph.D. who's constant kindness to the people he is working with, encyclopedia-like knowledge of immunology, great scientific experience and dedication to science made my years in his laboratory the most forming and unforgettable years of my professional life. I have been enjoying his countless merits and talents during the years he directed my research. They are the major reason why this thesis is materialized.

I would like to thank the Doctor of Science program at Boston University, Department of Environmental Health for extending my knowledge in the array of fields some of which I never really thought about before. Specifically, the education I have got at the program made me believe that molecular epidemiology (which is just occurring today) is one of the major directions which will influence science in the near future. The years in the program, and the courses and seminars I have attended made me also think about ethical dilemmas modern biology and biotechnology imposes.

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ORIGINAL COMMUNICATIONS

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1. K. Yamaguchi, R. Near, A. Shneider, H. Cui, and D.H. Sherr (1996) Fluoranthene-Induced Apoptosis in Murine T Cell Hybridomas is Independent of the Aromatic Hydrocarbon Receptor. Toxicology and Applied Pharmacology 139, 144-152
2. C. Vaziry, A. Shneider, D.H. Sherr, and D. Faller. (1996) Expression of the Aryl Hydrocarbon Receptor is Regulated by Serum and Mitogenic Growth Factors in Murine 3T3 Fibroblasts. The Journal of Biological Chemistry Vol. 271, No. 42, 25921-25927
3. K. Yamaguchi, R. Near, R. Matulka, A. Shneider, P. Toselli, A. Trombino, and D. Sherr. (1997) Activation of the Aryl Hydrocarbon Receptor/Transcription Factor and Bone Marrow Stromal Cell-Depandent pre-B Cell Apoptosis. 1997 The Journal of Immunology Vol. 158, No. 5, 2165-2173
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Submitted to Toxicology and Applied Pharmacology.
5. K. Yamaguchi, R.A. Matulka, A.M. Shneider, P. Toselli, A.F. Trombino, S. Yang, L.J. Hafer, K.K. Mann, J.L. Tilly, R.I. Near, D.H. Sherr (1997) Induction of Pre-B Cell Apoptosis by 7,12-Dimethylbenz[α]anthracene in a Long Term Bone Marrow Cultures. Toxicology and Applied Pharmacology 147, 190-203.

NOT INCLUDED IN THE THESIS:

6. R.I. Near, K.K. Mann, R.A. Matulka. A.M. Shneider, S.U. Golgate. A.F. Trombino, and D.H. Sherr (1997) Hepatic Parenchymal or Bone Marrow Stromal Cells Expressing the Aromatic Hydrocarbone Receptor Regulate Aromatic Hydrocarbone-Induced Apoptosis in Pre-B cells. Submitted to The Journal of Immunology

SUMMARY.

The aryl hydrocarbon (dioxin) receptor (AhR) is a transcription factor with no known endogenous ligands/stimuli and with poorly defined physiologic function(s). In the polluted environment, toxic compounds like polycyclic aromatic hydrocarbons (PAH) and related halogenated hydrocarbons (e.g. dioxins) activate the AhR leading to steroid-like immunosuppression, cell transformation, and clinical manifestations like carcinogenecity, terategenecity, and liver toxicity. Researchers infer a role for the AhR in cell growth and/or function, but the detailed mechanism(s) of the AhR functioning remains to be resolved. Exploration of intracellular signaling pathways regulated by AhR activation could provide mechanistic insights into PAH toxicity and AhR biochemistry.

I. NF-κB is a stress activated transcription factor expressing the genes coding for immuno-receptors, cytokines, and proto-oncogenes. Stimulation of NF-κB leads to a multitude of defense reactions among which modulation of apoptosis is a most recently described one. We postulated and tested the hypothesis that the AhR ligated with PAH activates cytoplasmic NF-κB. This suggests a number of new toxic outcomes for PAH exposures, and offers novel mechanistic explanations for the toxicity of the known compounds.

Murine hepatoma cells previously characterized for AhR and cytochrome P-450 activities were treated with PAH, and their binding activity to Igκ light chain promotor (a known NF-kB binding site) was tested by electrophoretic mobility shift assays. The results were: 1) NF-kB activity was induced in AhR^+ hepatoma cells following treatment with two tested PAH, benzo[α]pyrene (B[α]P), and 7,12-dimethylbenz[a]anthracene (DMBA); 2) PAH-induced NF-κB activation was prevented by pre-treatment of the cells with a known inhibitor of the AhR and P-450 activity, α-naphthoflavone (α-NF); 3) NF-κB was not induced by benzo[*e*]pyrene, a B[α]P stereo-isomer which has low affinity to the AhR and low bioactivity; 4) mutant hepatoma cell lines defective in AhR signaling machinery did not undergo NF-κB activation in response to PAH treatment; 5) a hepatoma mutant possessing reduced AhR-regulated P-4501A1 enzyme activity undergoes NF-κB activation in response to PAH treatment. These results demonstrate PAH-induced AhR-mediated NF-κB activation.

II. NF-κB seems to be just one of a number of signaling molecules communicating with the AhR, but a productive search for such molecules is impossible until we know at which stage(s) of a cell cycle the AhR is present and active. More over, indirect evidences suggest AhR involvement in cell growth and differentiation. Control of the intracellular AhR level by the mammalian cell cycle has not been adequately studied. In the present research, we addressed this question using the murine 3T3 fibroblasts model. Our results indicate that: 1) 3T3 cells growth-arrested by serum deprivation reduce the AhR level, and release from growth-arrest by restoring the serum concentration or supplementation with fibroblast mitogens (PDGF, bFGF) restores the AhR protein level; 2) expression of the AhR in synchronized 3T3 fibroblasts released from growth arrest corresponds to S-phase of the cell cycle (onset of DNA synthesis) reaching a peak at late S-phase; 3) serum-dependent regulation is executed via the AhR promoter as it is demonstrated by transient transfections with an AhR-promoter-luciferase

construct; 4) the G1 blocker, sodium-butyrate, prevents serum-stimulated cell progression through the cycle, but does not prevent expression of the AhR. These data suggests differential cellular response to PAH and other AhR ligands due to the difference in the AhR expression betwin cycling and resting cells. These data also support the notion that the AhR may be involved in the cellular proliferative responses.

III. The majority of PAH immunosupression studies investigate effects of PAH on mature B and T cells. There is not enough information about PAH toxicity to more sensitive immature lymphocytes. In the present study, we use DMBA as a prototype PAH to study it's effects on long-term primary as well as cloned bone marrow cultures consisting of immature preB cells maintained in a supportive microenvironment provided by bone marrow stromal cells. Our research demonstrate that: 1) doses of DMBA as low as 10^{-8} M induce pre B cell apoptosis in cloned and/or primary bone marrow cultures; 2) *in situ* hybridization, RT-PCR, and immunoblotting indicate that stroma cells, but not pre B cells, express AhR mRNA and protein; 3) immunohistochemistry reveals the presence of AhR protein in cloned stroma cells and freshly isolated unfractionated bone marrow cells, but not in purified preB cells; 4) rIL-7 added to the media supports growth of cloned preB cells, but fails to support DMBA-induced apoptosis; 5) α-NF prevents DMBA-induced preB cell apoptosis in primary bone marrow cultures. These results suggest that apoptosis is one of the mechanisms for PAH immunosupression. DMBA-induced preB cell death may result not from direct PAH interaction with preB cells, but indirectly from the changes in supporting environment due to the effects of PAH on AhR^+ bone marrow stromal cells.

IV. Fluoranthene is an environmentally ubiquitous but understudied PAH. We studied fluoranthene's potential to induce apoptosis in murine T cell hybridomas, and the molecular mechanism(s) of the apoptosis. The potential involvement of the AhR was under special scrutiny. Our results demonstrate that: 1) exposure to fluoranthene induce apoptosis within 8 h after treatment in three of four T cell hybridomas tested; 2) fluoranthene does not induce AhR nuclear translocation in the cells possessing high quantities of the AhR; 3) the AhR protein and/or mRNA were not found in fluoranthene-responsive T hybridomas; 4) fluoranthene-induced apoptosis is significantly reduced by treatment with EGTA, Ca^{2+} chelating agent. These data demonstrate the immunosuppressive potential of fluoranthene. They also extend to the T cell compartment the notion that apoptosis is one of the mechanisms for PAH-induced immunotoxicity. Finally, fluoranthene-mediated T cell apoptosis is AhR-independent. As a result, risk assessment should take in into account both AhR dependent and independent mechanisms of PAH toxicity.

SUMMARY IN DANISH (SAMMENDRAG)

Aryl hydrocarbon (dioxin) receptoren (AhR) er en transkriptionsfaktor, der ikke har nogen kendte endogene ligander/stimuli og som er dårligt beskrevet mht. fysiologiske funktion(er). I forurenede miljøer vil toksiske forbindelser såsom polycykliske aromatiske hydrocarboner (PAH) og relaterede halogenerede hydrocarboner (f.eks. dioxiner) aktivere AhR, hvilket medfører steroid-lignende immunosuppression, celle-transformation og kliniske manifestationer som f.eks. carcinogenitet, teratogenitet og lever-toksicitet. Tidligere studier har indikeret at AhR spiller en rolle med hensyn til cellevækst og/eller funktion, men de(n) detaljerede mekanisme(r) bag AhRs funktion kendes endnu ikke. Undersøgelser af de intracellulære signaleringsveje, der reguleres af AhR aktivering, kan give mekanistisk indsigt i PAH toksicitet og AhRs biokemi.

I. NF-κB er en stressaktiveret transkriptionsfaktor, der medfører ekspression af gener der koder for immunoreceptorer, cytokiner og proto-oncogener. Et større antal forsvarsreaktioner vil blive aktiveret ved NF-κB stimulering. Herunder modulering af apoptose som nyligt beskrevet. Vi postulerede og testede hypotesen at AhR med PAH bundet aktiverer cytoplasmisk NF-κB. Dette skulle medføre adskillige hidtil ukendte toksiske effekter af PAH eksponering og en ny mekanistisk forklaring på PAH toksicitet.

Murine hepatomaceller, der tidligere er blevet karakteriseret for AhR og cytochrom P-450 aktiviteter, blev eksponeret til PAH, og deres bindingsaktivitet til Igκ light chain promotor (et kendt NF-κB binding site) blev testet ved brug af 'electrophoretic mobility shift assays'. Resultaterne viste at: 1) NF-κB aktiviteten blev induceret i AhR^+ hepatomaceller efter eksponering til to testede PAHer, benzo[α]pyren (B[α]P) og 7,12-dimethylbenz[a]anthracen (DMBA); 2) PAH-induceret NF-κB aktivering blev forhindret ved forbehandling af cellerne med en kendt inhibitor af AhR og P-450, α-naphthoflavon (α-NF); 3) NF-κB blev ikke induceret af benzo[*e*]pyrene, en B[α]P stereo-isomer med lav affinitet overfor AhR samt lav bioaktivitet; 4) Mutante hepatoma celle linier, med defekt i Ahr signaleringsfunktion, aktiverede ikke NF-κB afhængige processer som respons på PAH eksponering; 5) En hepatoma mutant, med reduceret AhR-reguleret P-4501A1 enzymaktivitet udviste NF-κB aktivering ved PAH eksponering. Resultaterne viser PAH-induceret AhR-medieret NF-κB aktivering.

II. NF-κB er tilsyneladende kun én ud af et antal af signalmolekyler, der kommunikerer med AhR, men effektiv søgning efter sådanne molekyler er umulig indtil vi ved i hvilke stadie(r) af celle-cyklus AhR er tilstede og er aktiv. Ydermere er der indirekte beviser for at AhR er involveret i cellevækst og differentiering. Kontrol af det intracellulære AhR niveau via pattedyrs celle-cyklus er ikke blevet undersøgt tilstrækkeligt. Vi forsøgte at besvare dette spørgsmål ved at anvende 3T3 fibroblast modellen. Vores resultater indikerer at: 1) 3T3 celler, der blev vækst-standset ved serum-mangel reducerede AhR niveauet. Frigivelse fra vækst-standsning (ved tilsætning af serum eller fibroblast mitogener (PDGF, bFGF)) gendanner AhR proteinniveauet; 2) Ekspression af AhR i synkroniserede 3T3 fibroblaster frigivet fra vækst-standsning svarer til S-fasen i cellecyklus (start af DNA syntese) med et maximum den sene S-fase; 3) serum-afhængig regulering udføres via en AhR promotor som vist ved transient transfektion med en AhR-promotor-luciferase konstrukt; 4) G1 blokkeren, natrium-

butyrat, forhindrer serum-stimuleret celleprogression gennem cyklus, men forhindrer ikke ekspression af AhR. Disse resultater indikerer, at der er tale om et differentieret cellulært respons til PAH og andre AhR ligander pga. forskellene i AhR ekspression mellem voksende og hvilende celler. Endvidere understøtter resultaterne at AhR kan være involveret i celleproliferations respons.

III. De fleste PAH immunosupressions-studier har undersøgt PAH effekter på modne B- og T-celler. Der mangler information om PAH toksicitet overfor mere følsomme umodne lymfocytter. I nærværende studie blev DMBA anvendt som prototype PAH ved undersøgelser af effekter på langtids primære og klonede knoglemarvskulturer bestående af umodne præB-celler holdt i understøttende mikromiljøer dannet af knoglemarvs stromaceller. Vores forsøg viste at: 1) DMBA koncentrationer helt ned til 10^{-8} M inducerede præB-celle apoptose i klonede og/eller primære knoglemarvskulturer; 2) *In situ* hybridisering, RT-PCR, og immunoblotting indikerede at stromaceller, men ikke præB-celler, ekspresserer AhR mRNA og protein; 3) Immunohistokemiske forsøg angav tilstedeværelsen af AhR protein i klonede stromaceller og nyligt isolerede ikke-fraktionerede knoglemarvsceller, men ikke i oprensede præB-celler; 4) rIL-7 tilsat til mediet understøtter væksten af klonede præB-celler, men ikke DMBA-induceret apoptose; 5) α-NF forhindrer DMBA-induceret præB-celle apoptose i primære knoglemarvskulturer. Disse resultater indikerer, at apoptose er en mekanisme for PAH immunosupression. DMBA-induceret præB-celledød er ikke et resultat af direkte PAH interaktion med præB-celler, men derimod et indirekte resultat af ændringen i det understøttende miljø pga. effekterne af PAH på AhR^+ knoglemarv stromaceller.

IV. Fluoranthen er en miljømæssigt udbredt men dårligt undersøgt PAH. Vi undersøgte fluoranthens potentiale for at inducere apoptose i murine T-celle hybridomer og de(n) molekylære mekanisme(r) for apoptose med særligt fokus på AhRs mulige betydning. Vores resultater demonstrerede at: 1) I tre ud af fire testede T-celle hybridomer blev apoptose induceret indenfor 8 timer efter flouranthen eksponering; 2) Fluoranthen inducerede ikke kernetranslokation af AhR i de celler der indeholdt store mængder AhR; 3) Hverken AhR protein og/eller mRNA blev fundet i fluoranthen-responsive T-celle hybridomer; 4) Fluoranthen-induceret apoptose blev signifikant reduceret ved behandling med EGTA, et Ca^{2+} kelaterende stof. Resultaterne indikerer at flouranthen er potentielt immunosuppressiv. De viser endvidere at apoptose er en mekanisme for PAH-induceret immunotoksicitet i T-celler. Ydermere er fluoranthen-medieret T-celle apoptose uafhængig af AhR. På denne baggrund foreslås det både at indrage de AhR-afhængige og uafhængige mekanismer i risikovurdering af PAH toksicitet.

INTRODUCTION.

The aryl hydrocarbon (dioxin) receptor.

A number of hydrophobic hormones and extracellular signaling molecules signal through specific protein receptors anchored in cytoplasm and translocating to the nucleus upon ligation. The aryl hydrocarbon (dioxin) receptor (AhR) is an example for this class of receptors (Cuthill, Poellinger, and Gustafsson, 1987; Dolwick, Swanson, and Bradfield, 1993; Ema *et al.*, 1992). In the cytoplasm, AhR is associated with heat shock protein 90 which may participate in ligand binding and, possibly, with other poorly characterized proteins. The AhR nuclear translocation factor (ARNT) (Perdew, 1988) is another accessory molecule which dimerizes with ligated AhR to form a transcription factor inducing gene transcription via binding to the xenobiotic-specific DNA response elements (XRE) and recruiting other transcription factors to the promoter (Poland and Knutson, 1982). AhR and ARNT associate via helix-loop-helix motifs present in both monomers (Reyes, Reisz-Porszasz, and Hankinson, 1992). For some genes AhR is not an expression but a repression factor which binds XRE overlapping other positive regulatory elements (Krishnan *et al.*, 1995). Despite a lot of effort to find an endogenous, "physiologic", ligand for AhR, such a factor was not determined, but multiple potent ubiquitous environmental pollutants, including polycyclic aromatic hydrocarbons (PAH), polychlorinated biphenyls, and halogenated aromatic hydrocarbons (e.g. dioxins) activate the AhR. (Poland and Knutson, 1982).

Benzo[α]pyrene (B[α]P) and/or 7,12-dimethylbenz[a]anthracene (DMBA) are typical examples of PAH, and 2,3,7,8-tetrachlorodibenzo-p-dioxin or TCDD is the most commonly used of halogenated aromatic hydrocarbon (HAH). PAHs are byproducts of combustion of fossil fuels and tobacco smoking. They are also present in some types of traditional food like smoked meats. TCDD and other HAHs contaminate manufactured chlorophenols and other commodities.

Molecular and cellular responses to PAH or TCDD induced AhR activation may suggest the AhR function(s). For example, a role for the AhR in cell growth and function may be inferred from the fact that activated AhR induces PAH-specific cytochrome P-450 genes (Cuthill, Poellinger, and Gustafsson, 1987; Holsapple *et al.*, 1991), activates *c-Ha-ras, c-myc* and *c-erb*-A proto-oncogenes (Sadhu *et al.*, 1993; Bombick *et al.*, 1988), modulates glucocorticoid, epidermal growth factor, estrogen and progesterone receptors (Safe and Krishnan, 1995; Abbott *et al.*, 1994), and induces cyclin-dependent and protein tyrosine kinases (Ma and Babish, 1993; Enan and Matsumura, 1995). A role for the AhR in lymphocyte development and function is anticipated because of the ability of these exogenous AhR ligands to modify lymphocyte signaling (Davila *et al.*, 1995; Karras and Holsapple, 1994), to induce IL- 1β, TGF-α, TGF-β gene transcription (Sutter *et al.*, 1991; Choi *et al.*, 1991; Gaido *et al.*, 1992), and to modulate both T and B cell responses (Cuthill, Poellinger, and Gustafsson, 1987; Harper *et al.*, 1995; Hardin, Hinoshita, and Sherr, 1992; Kawabata and White, 1987; Silkworth and Vecchi, 1985; Blank *et al.*, 1987; Kerkvliet *et al.*, 1990; Wojdani *et al.*, 1984; Morris *et al.*, 1994; Fine, Silverstone, and Gasiewicz, 1990; Thurmond *et al.*, 1988; White and Holsapple, 1984). Hypothesis that

the AhR is involved in lymphocyte development is also supported by the fact that lymphocyte development is impaired in AhR gene knock-out mice (Fernandez-Salguero *et at.*, 1995).

The "natural" function(s) of the AhR remains to be revealed, although the major focus of current studies is to reveal the role of AhR in the pollution induced toxicity. Contrary to the dominant point of view in the field, the author thinks that the reason why the internal AhR ligand was not found may be not a temporary luck of success or technical constrains in the search for the endogenous ligand but the absence of such a "natural" ligand in the real life. Basically, the notion that endogenous ligand does exist came from the fact that AhR was originally isolated as a protein binding with high affinity industrially produced environmental pollutants and, consequently, the protein was named "a receptor". Industrially produced chemicals can mimic but can not be a physiological ligand. So, what is one?

The author believes that this way of thinking represents psychological inertia rather than a solid basis to expect an endogenous ligand. From another hand, there could be some posttranslational modifications that change the three-dimensional conformation of the AhR, bringing the protein to the active form. It could be hypothesized that these posttranslational modifications are the real natural AhR inducers. Upon binding to the AhR, xenobiotic ligands could induce conformational changes mimicking ones induced by the physiological posttranslational modifications. The history of biochemistry offers at least one family of receptors which for decades were believed to function only as sensors for their ligands, but now it appears that ligation is just one of the ways to activate them as the transcription factors. That is the steroid hormone receptor family. Despite the lack of sequence homology, AhR and steroid hormone receptors share such features as association with hsp 90, conversion of the receptor into a transcription factor which induces enzyme and growth factor genes, the ability of receptor ligands to act as immunosuppressants and apparent contributions to cell growth and death.

Nuclear factor-κB.

There is not much known today about the interaction of the AhR with other transcription factors (Sadek and Allen-Hoffmann, 1994). Continuing the analogy with steroid receptors- some of which exhibit cross-talk with the ubiquitous transcription factor NF-κB- it would be of interest whether the AhR interacts with NF-κB as well. In cytoplasm, NF-κB (which is a homo- or heterodimer of p50, p52, p55, RelA, c-Rel, or Rel B constituting NF-κB/Rel family) is anchored by an inhibitor called IκB (Baeuerle and Henkel, 1994; Miyamoto and Verma, 1995; Phillips, Gustafson, and Ghosh, 1996). NF-κB activators induce IκB phosphorylation followed by a proteolytic digestion of the inhibitor, and NF-κB nuclear translocation. In the nucleus, NF-κB binds to specific decamer sequence in the enhancer and/or promoter region of the responsive genes and induces their transcription. The incomplete list of NF-κB regulated genes consists of immuno-receptor genes (e.g. Igκ light chain, T-cell receptor-b chain, IL-2 receptor a-chain, MHC class I, b2-microglobulin), cytokine genes (e.g. β-interferon, GM-CSF, G-CSF, interleukin-6, interleukin-2, TNF-α), a proto-oncogene (*c-myc*), and viral genes

(e.g. HIV LTR, cytomegalovirus immediate-early gene US 3) (Lenardo, Pierce, and Baltimore, 1987; Jamieson, Mauxion, and Sen, 1989; Leung and Nabel, 1988; Baldwin Jr. and Sharp, 1988; Visvanathan and Goodbourn, 1989; Schreck and Baeuerle, 1990; Lee *et al.*, 1995; Nabel and Baltimore, 1990; Thrower *et al.*, 1996; Baeuerle and Henkel, 1994). NF-κB activation changes cell growth (Miyamoto and Verma, 1995), and prevents apoptosis, at least in B cells (Wu *et al.*, 1996; Arsura, Wu, and Sonenshshein, 1996; Schauer *et al.*, 1996).

The list of NF-κB stimuli include physical hazards, like UV radiation, chemical toxins like H_2O_2, viruses and their double stranded RNA, and ligation of receptors such as Fas, TNF-α, CD40, immunoglobulin, antigen-specific T cell receptors, glucocorticoid receptor, and IL-1 cytokine receptor (Miyamoto and Verma, 1995; Verma *et al.*, 1995; Olashaw *et al.*, 1992; Auphan *et al.*, 1995; Freimuth, Depper, and Nabel, 1989; Griffin *et al.*, 1989). All the obviously distinct NF-κB activators share an induction of oxidative stress as a common element of NF-κB stimulation (Schreck, Albermann, and Baeuerle, 1992). As was shown recently *in vitro* on the NF-κB-responsive promoter reporter plasmid construct (Yao *et al.*, 1995) and *in vivo* with thymic NF-κB induction (Olnes, Verma, and Kurl, 1994), TCDD activates NF-κB. It raises the question whether nonhalogenated PAH induce NF-κB as well.

In the present study, the hypothesis was postulated and tested that PAH induced AhR activation would lead to NF-κB induction. Three lines of evidence supported this hypothesis. First, the AhR has a functional similarity to steroid receptors modulating NF-κB (Auphan *et al.*, 1995). Second, the AhR induced cytochromes P-450 metabolize nonhalogenated PAHs to the oxidatively reactive intermediates which may predispose a cell to the state of oxidative stress. Third, there is a remarkable overlap in the cellular changes induced by the AhR and NF-κB activation, for example, altered cytokine production, immunosuppression (Faith and Moore, 1977; Faith, Luster, and Moore, 1978; Vos and Moore, 1974), and the induction of programmed cell death (Gao *et al.*, 1996; Burchiel *et al.*, 1993; Yamaguchi *et al.*, 1996; Yamaguchi *et al.*, 1997). The hypothesis was tested by assessing NF-κB activity in vehicle, B[α]P or DMBA treated Hepa-1c1c7 (Hepa-1) liver cell lines expressing high AhR levels (Hankinson, 1983). To address the role of the AhR and P-4501A1 enzymes in PAH-induced NF-κB activation, Hepa-1 cell line was compared with it's mutants defective in the AhR, ARNT or AhR-regulated P-4501A1. The results herein support the idea that activated AhR induces NF-κB, and raise the question which of toxic outcomes of PAH exposure could have this activation as a molecular mechanism.

A role of the stroma cells as mediators of PAH-induced pre B cells apoptosis is describe below. Hepa-1 cells could serve as stroma supporting both growth and PAH-induced apoptosis of pre B cells (R. Near *et al*, submitted). The presented data raises the question if NF-κB activation mediates the process.

Cell cycle is AhR expression regulating factor.

One of the major goals of modern AhR research is to understand the mechanism(s) and factors which regulate the AhR expression. Indeed, difference in the AhR expression could account for cell-specificity in the toxicological responses to PAH. Another reason for the interest is that the AhR may be physiologically involved in normal growth and differentiation, and that toxicological induction of the AhR signal transduction jeopardizes normal development. The supporting evidences are that PAH induce increased cell proliferation (e.g. parenchymal cells, epithelial cells of the urinary tract, the intestine, and the interfollicular epidermis), inhibition of differentiation, as well as tumor promotion in experimental animals (Poland and Knutson, 1982, and references therein). In order to understand the natural involvement of the AhR in growth and development one has to know what regulates the AhR expression. In the present study we reveal that expression of the AhR is under control of the mammalian cell cycle. This data supports the idea that the AhR is involved in the cell cycle and proliferation.

Multiple cell cycle studies and research on signal transduction regulated by growth factors were performed on murine 3T3 fibroblasts. These immortal, non-transformed cell lines are derived from mouse embryos (Pardee, 1991). The 3T3 fibroblasts system is extensively described for it's growth properties, growth factors involved, genes and biochemical pathways controlling the cell cycle. 10% donor calf serum is added to the media as the standard source of growth factors supporting exponential growth of 3T3 fibroblasts. Reduction of the calf serum concentration down to 0.5% arrests cell growth holding the cells in G_0, the quiescent phase of a cell cycle. The cell cycle phase positioned between G_0 (or mitosis) and the initiation of DNA synthesis (S-phase) is called G_1 phase. Rescue of cells from G_0 to G_1 phase of a cell cycle may be achieved by stimulation with mitogenic growth factors like PDGF and/or bFGF, or by restoring serum level back to 10%. This is one of the major models in cell synchronization experiments and studies of G_1-specific growth factor-stimulated signaling events such as growth factor-induced synthesis of second messenger molecules, activation of small GTP-binding proteins, protein kinase cascades, and the transcriptional activation of immediate-early and delayed-response genes coding for proteins suspected to be involved in progression through the cell cycle (reviewed in references Pardee. 1991). Overall, 3T3 fibroblasts provided a valuable tool which has been used to reveal molecules involved in or controlled by execution of a cell cycle program stringently regulated by external mitogenic stimuli.

In the present study, 3T3 fibroblast were used as a model to investigate expression of the AhR during the cell cycle. We demonstrated the regulation of the AhR transcription by serum and purified growth factors during the course of the cell cycle. From the toxicological point of view, this data suggests the stages of the cell cycle when the cell is the most susceptible to the environmental toxins acting through the AhR. From the physiological point of view, our results support the notion that the AhR is involved in normal cell growth and development.

Immunotoxicity of PAH.

Although the majority of toxicological research was dedicated to PAH-induced cell transformation, there are a number of studies demonstrating the immunosuppressive potential of PAH (Burchiel *et al.* 1993; Burchiel *et al.* 1992; Davila *et al.* 1995; Hardin *et al.* 1992; Hinoshita *et al.* 1992; Kawabata and White 1987; Thurmond *et al.* 1988; White and Holsapple 1984; White *et al.* 1985; Wojdani *et al.* 1984; Yamaguchi *et al.* 1996; Temple *et al.* 1993). Immunosuppressive effects of PAH in humans are suggested from epidemiological studies (Hoffman *et al.* 1986; Kimbrough 1987; Szczeklik *et al.* 1994), and from the animal model systems where exposure to TCDD, DMBA, B[α]P, fluoranthene, and polychlorinated biphenyls (PCBs) may lead to decreased resistance to infectious agents and transplantable tumors. Some of the cellular and molecular mechanisms of PAH immunotoxicity are reduced bone marrow cellularity, inhibited B cell antibody responses, thymic atrophy, altered lymphocyte homing, impaired B and T lymphocyte proliferation, reduced natural killer activity, decreased cytotoxic T cell activity, induced cell death in myeloid, B and T cells, and decreased cytokine production (Davis and Safe, 1991; Dooley and Holsapple 1988; Fine *et al.* 1990; Gasiewicz and Rucci 1991; Greenlee *et al.* 1985; Harper *et al.* 1995; Holladay and Smith 1995; Holsapple *et al.* 1991; Kremer *et al.* 1994; Morris *et al.* 1994; Wood *et al.* 1993; Ackerman *et al.* 1989; Fine *et al.* 1990; Kerkvliet *et al.* 1990; Silkworth *et al.* 1984; Burchiel *et al.*, 1992; White and Holsapple, 1984; Karras and Holsapple,1994).

The spectrum of PAH immunotoxic outcomes suggests that PAH interfere with lymphocyte programmed cell death/apoptosis machinery. Apoptosis is a tightly regulated sequential process of cell suicide evident by cell shrinkage, increase of ruffness of cell membrane, loss of cell to cell contact, DNA digestion to oligonucleosome-sized (185 bp) fragments, chromatin condensation, and cell disintegration to membrane covered particles which are readily phagocytised by surrounding cells. Any disregulation of apoptosis may lead to clinical manifestations (e.g. inappropriate activation of apoptosis may result in immunodeficiency and/or neurodegenerative disorders, and cell inability to apoptose could lead to cancer). It was shown for a diverse array of apoptosis modulating stimuli that they prevent formation of normal lymphocyte repertoire (Ju *et al.*, 1995; Gillette-Feruson and Sidman, 1994), overwhelm immune response, and/or lead to autoimmunity by reducing peripheral tolerance (Ucker *et al.*, 1992).

The actual molecular mechanism of PAH immunotoxicity is not yet known. Some research indicates that AhR and/or PAH metabolism may be involved (Hardin *et al.* 1992; Holsapple *et al.* 1991; Kerkvliet *et al.* 1990; Ladics *et al* 1991; Morris *et al.* 1994; Silkworth *et al.* 1984; Thurmond *et al.*, 1988; White *et al.* 1985; Harper *et al.* 1994), but these studies did not address the issue of whether PAH activate AhR directly in lymphocytes or in stroma cells supporting lymphocytes. Indirect mechanism may be inferred from experiments on mature T cell populations and clones, and on immature lymphocyte populations (Kremer, Gleichmann, and Esser, 1994; Greenlee *et al.*, 1985).

Despite the well-accepted opinion that environmental pollutants are especially damaging for developing biologic systems (Holladay and Smith, 1995), there are few studis on the effects of PAH on developing immune systems (Morris *et al.* 1994; Blaylock *et al.* 1992; Luster *et al.* 1988). It was shown previously that DMBA suppresses

activity of mature lymphocyte *in vivo* and *in vitro* (Holladay and Smith, 1995; Burchiel *et al.*, 1992; Burchiel *et al.*, 1993; Davila *et al.*, 1995; Ladics *et al.* 1991). In our study we tested DMBA for it's ability to affect B cell lymphopoiesis in an *in vitro* model.

The *in vitro* model originally developed by Whitlock and co-authors (Whitlock *et al.* 1984) represents an *in vivo* preB lymphocyte development in the way that long-term pre B cells grow on top of, and in tight contact with a supporting stroma cell line. The stroma cells provide all the factors necessary for lymphocyte growth and development. When the Whitlock cell culture system is treated with DMBA, the toxicity may result from PAH effects on pre B lymphocytes per se, or from DMBA induced alteration(s) of stroma cell support (or both). We investigated whether DMBA induces pre B cell apoptosis directly or by altering stroma cell metabolism and the support provided by stroma cells. Exploring the biochemical mechanism(s), we focused on DMBA metabolism and involvement of the AhR as a possible mediator of DMBA immunotoxicity.

Another nonhalogenated PAH which is not as extensively studied as DMBA or B[α]P is fluoranthene. In our study we tested fluoranthene for its ability to induce apoptosis in T cells. The need for this research came from a growing body of evidence that nonhalogenated PAH induce apoptosis in T and B cells *in vitro* and *in vivo* (Burchiel *et al.*, 1992, 1993; Hardi *et al.*, 1992; Hinoshita *et al.*, 1992), and from especial importance of fluoranthene.

Fluoranthene is wide-spread in environment, and is often associated with other PAH in complex mixtures (IARC, 1983). Simultaneous exposure to fluoranthene and other PAH may lead to synergism of toxic outcomes like immunosupression and/or cell transformation. Fluoranthene (mutagenic by itself (Wang and Busby, 1993)) potentiates the carcinogenicity of B[α]P (Rice *et al.*, 1984). Fluoranthene shares structural similarities with other immunotoxic PAH, so it is not surprising that fluoranthene suppresses B lymphopoiesis (Hinoshita *et al.*, 1992).

Our goal was to study if fluoranthene induces apoptosis in T lymphocytes (the majority of the fluoranthene research was previously focused on B cells), and to discover the intracellular signals involved in PAH-induced apoptosis. PAH treated T cell hybridomas were used as a model. As was discussed above, the AhR may be controlled by and involved in the cell cycle. Apoptosis events are also, in many cases, connected to a cell cycle program. We analyzed the effect(s) of fluoranthene treatment on T hybridoma cell cycle. A possible role of the AhR in PAH-induced T cell apoptosis was investigated due to the common expectation that the AhR is a major mediator of any PAH toxicity. A possible role of extracellular Ca^{2+} ions was also under study because their involvement was anticipated by analogy with antigen-specific receptor-mediated apoptosis.

MATERIALS AND METHODS.

Cell culture conditions: Cloned bone marrow stromal cells, BMS2, which support pre-B cell growth were generously provided by Dr. P. Kincade (Oklahoma Medical Research Foundation) and have been previously described (Pietrangeli *et al.*, 1988). Hepa-1 (clone Hepa-lclc7) cells were generously provided by Dr. J. Whitlock (Stanford

University). BMS2 cells, wild type and mutant Hepa-1 cells were grown at 37° C, 10% CO_2 in DMEM supplemented with 10% bovine fetal serum (FCS) (GIBCO-BRL Life Technologies, Inc. Grand Island, NY), 100 units/ml penicillin and streptomycin, 1mM glutamine, and 10^{-5} M β-mercaptoethanol. Cells were split 1:8 three times a week. To assess NF-κB activation, cultures were treated for one hour with vehicle (acetone) or 10^{-5}-10^{-8} M B[α]P or DMBA (Sigma, St. Louis, MO) dissolved in acetone (final vehicle concentration was 0.1%) in duplicate flasks. Vehicle had no effect on cultures.

Swiss 3T3 cells were obtained from ATCC and maintained similar to Hepa-1 cells. To induce growth arrest of Swiss 3T3 fibroblasts, cells were placed in culture medium containing 0.5% FCS for 24 hours.

Murine bone marrow cultures were prepared from C57BL/6 (AhR^b) bone marrow as described (Whitlock *et al.* 1984). Briefly, bone marrow was expunged from the femurs of 3-4 week old mice, washed, counted, and cultured in RPMI medium containing 5% FCS (Gibco/BRL, Inc., Grand Island, N.Y), 2 mM L-glutamine (Gibco/BRL), 50 U/ml penicillin/streptomycin (Gibco/BRL), and 0.05 mM 2-mercaptoethanol (Mallinckrodt, Paris, KY) (3 x 10^6 cells/3 ml in 6 well plates). One half of the medium was replaced every 3-4 days with fresh medium. Growth of stromal cells and stromal cell-adherent lymphocytes was routinely evident after 10 days. After four weeks of culture, >90% of stromal cell-adherent cells consisted of $B220^+$, $sIgM^-$ B lymphocytes as assessed by flow cytometry (see below) with rearranged immunoglobulin heavy chains. 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. At various points thereafter pre B cells from duplicate wells were pooled and assayed for apoptosis. Vehicle had no effect on cultures.

Derivation and DMBA treatment of cloned pre B cell lines was done as following. After 4 weeks 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 pre B lymphocyte growth (Pietrangeli *et al.*, 1988). Transferred lymphocytes readily adhered to BMS2 cells and lymphocyte growth was evident within 3 weeks. Resulting cell lines, one of which is referred to as BU-11, were maintained and treated as described for primary bone marrow cultures. The BU-11 cell line has been maintained for over 1 year 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]F1; AhR^{b-1}/AhR^d respectively) expressing relatively high levels of high affinity AhR (Chang *et al.*, 1993). 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-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.

Hybridomas ME1 and K3lH28 were derived by fusing sperm whale myoglobin-immunized Balb/c lymph node cells and keyhole limpet hemocyanin-immunized A/J

lymph node cells respectively with the HGPRT- BW5147 fusion cell line. Hybridomas 5D5 and 12.13 were produced from separate fusions of BW5147 cells and cultured lymph node cells from MRL-lpr/lpr mice (Ju *et al.,* 1995). Fusions were selected in HT containing medium and were cloned by limiting dilution. Hybridomas were routinely maintained in DMEM supplemented with 10% FCS, 100 u/ml penicillin and streptomycin, 1 mM L-glutamine (Gibco-BRL, Inc., Grand Island, NY), and $5x10^{-5}$ M 2-mercaptoethanol (Sigma). All hybridomas expressed high levels of the T cell receptor as assessed by flow cytometry. Hybridoma T cells were maintained in log growth phase by splitting cultures 1: 3 every day. Cells (10^5) were added to 5-cm^2 culture wells in 1 ml medium. Vehicle (acetone) or fluoranthene dissolved in acetone was added (final vehicle concentration =0.1 %) and cultures were harvested at various points thereafter for apoptosis or cell cycle assays. Vehicle had no effect on cultures.

Cytosolic protein: Hepa-1 monolayers (approximately 5 x 10^6 cells) were released from culture flasks by treating for 3 min. with 0.25% trypsin/1mM EDTA*4Na (Life Technologies, Inc.). Cells were washed, re-suspended in lysis buffer (1% Triton X-100,150 mM NaCl, 25 mM Tris/HCl, 1 ug/ml aprotenin, 10 ug/ml leupeptin, 1 mM EDTA, 50 mM NaF, 1 mM orthovanadate, 1 mM PMSF) and nuclei removed by micro-centrifugation for 15 seconds at 15,000 x g. Supernatants containing cytosolic proteins were frozen at -20° C until use.

Nuclear protein isolation (this technique was developed by A.M.Sh)*:* Cell monolayers were lifted and washed as described above. Cells were pelleted, washed, and resuspended in 1 ml $P_{10}EG$ buffer (10% glycerol, $KH_2PO_4*3H_2O$ 5.7 mM, EDTA 7.5mM, pH 7.4) containing 0.2% Triton X-100 for Hepa-1 cells. Cell suspensions were rigorously pipetted. Nuclei were centrifuged for 12 min at 6,000 x G. $P_{10}EGD$ buffer/Triton X-100 treatment was repeated until microscopy confirmed a pure preparation of nuclei. Pellets of nuclei were washed twice with $P_{10}EGD$, resuspended in nuclei buffer (20 mM Hepes pH 7.9, 210 mM NaCl, 0.75 mM $MgCl_2$, 0.1 mM EDTA, 20% glycerol, 0.5 mM DTT and 0.5 mM PMSF added immediately before use) and microcentrifuged for 15 minutes at 15,000 x g. Cytosols were frozen at 20° C until use.

Western immunoblotting: Cell suspensions were washed twice in cold PBS, resuspended in lysis buffer and centrifuged for 15 min at 15,000 x g. Supernatant protein concentrations 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% polyacrylamide SDS gels. Electrophoresis was carried out at 150 volts for 1 hour. Proteins were transferred to nitrocellulose filters (Bio-Rad, Hercules, CA) at 150 milliamps for 1 hour or at 30 milliamps 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% nonfat dry milk, washed twice for 5 min in TBST, and incubated with monoclonal anti-AhR antibody Rpt1 (Singh and Perdew, 1993) at a 1:10,000 dilution for 1 hour at room temperature. Rpt1 antibody was a kind gift of Dr. Perdew. Filters were washed with TBST and incubated for 1 hour at room temperature with a 1:6,000 dilution of HRP-goat anti-mouse antibody (Sigma). Filters were washed twice and developed by chemiluminescence (Du Pont NEN Research Products Co., Boston, MA).

Electrophoretic gel mobility shift assay (EMSA): EMSAs were used to assess binding of constitutive or activated NF-κB to a ^{32}P-labeled, 84 base pair double stranded DNA probe containing the 5'-GGGACTTTCC-3' sequence from the Igk light chain enhancer. The pUC13 plasmid containing the NF-κB oligonucleotide was kindly provided by S.-T. Ju and T. L. Rothstein (Boston University). End-labeled DNA probes were generated by Klenow fill-in of 5' overhangs with ^{32}P-dATP. The reaction mix (2.5 ml 1 M Tris pH7.6, 2.5 ml 1M $MgCl_2$, 5 ml of 6000 Ci/mM ^{32}P-dNTPs, 1 ml Klenow enzyme, 500 ng of oligonucleotide probe, and double distilled water to bring the total volume to 26 ml) was incubated for 30 min. at 37° C. Labeled DNA was purified with Centri-Spin-20 columns (Princeton Separations, Adelphia, NJ) according to the manufacturer's instructions. Specific activity was determined and the volume adjusted with TE buffer to a final activity of 5,000 cpm/ml.

Nuclear protein (1 mg) was mixed with nuclei buffer to make a total volume of 5 ml, the volume of ^{32}P-labeled DNA probe containing 10,000 Cerenkov cpm, and the volume of double distilled water to bring the total mixture volume to 15 ml. The mixture was incubated at room temperature for 20 min., and run 1 hour at 100 V in a 5% polyacrylamide non-denaturing gel. The gel was dried, and put on x-ray film with intensifying screens for overnight exposure at -80° C.

To evaluate XRE binding activity in the nuclei fraction protein of Swiss 3T3 cells EMSA assay was performed as described above but ^{32}P-XRE probe was used instead of NF-κB one.

EMSA specificity was confirmed by addition of a 100-fold excess unlabelled NF-κB probe or irrelevant 32 base pair oligonucleotide 5'-TTTTTGAGCTCGGAGTTGCGTGAGAAGAGCCGGAGCCGGATC-3' containing the AhR XRE (Elferink, Gasiewicz, and Whitlock, 1990).

Annealing of sense and anti-sense oligonucleotides to make AhR probes was performed by mixing of equal volumes of sense- and anti-sense oligonucleotides in TE buffer (Tris 10mM, pH 7.6; EDTA 1mM pH 8.0), incubating the mix at 95° C for 10 min., and slowly cooling to room temperature. DNA concentration was adjusted to 1 mg/ml.

Ethoxyresorufin-o-deethylase (EROD) activity assay: Cytochrome P-4501A1 (EROD) activity was assessed as previously described (Hahn, 1993). Briefly, cells were cultured in 48-well plates and treated for the specified amount of time with a specific PAH. Shortly before assay, cells were rinsed with cold PBS to remove media. A mixture was made so the final reaction contained 1.0 mM NADPH, 10μM dicumarol, 1.5mM salicylamide, and 2uM 7-ethoxyresorufin in a 50mM Tris-NaCl buffer. Immediately before reading, the reaction mixture was added to all wells except the blank well which received all reagents except substrate. Plates were read in a Cytofluor 2000 fluorimeter (Millipore, Bedford, MA) every 2 minutes for 20 minutes using an excitation wavelength of 530 nm and emission wavelength of 590 nm. Results were compared to a standard curve of resorufin in 50mM Tris-NaCl buffer. All chemicals for EROD assays were purchased from Sigma Chemical Co.

Fluorescence analyses and sorting: PreB cells were harvested by gently washing 3-4 week old primary bone marrow cultures with media. To assess purity, preB cells were incubated for 40 minutes on ice with phycoerythrin-anti-CD45/B220 (clone RA3-6B2, rat

IgG 2a, Pharmingen) or with a phycoerythrin-labeled isotype control. Cells were washed and analyzed in a Becton/Dickinson FACScan flow cytometer. To sort CD45/B220$^+$ preB cells from bone marrow cultures or to sort bone marrow B cells from freshly isolated bone marrow, 10^6 cells were incubated as above with PE-anti-CD45/B220 or PE-conjugated isotype control antibody, washed in medium containing 20% FCS, and sorted with a Becton/Dickinson FACStar on the basis of lymphocyte morphology (forward and side light scatter parameters) and expression of CD45/B220. Sorted cells were re-analyzed after sorting and consisted of >95% CD45/B220$^+$ cells.

Quantitation of apoptotic cells was performed as previously described (Hardin *et al.* 1992; Hinoshita *et al.* 1992; Yamaguchi *et al.* 1996). Cells were washed in cold PBS, pelleted, and resuspended in 0.5 ml of hypotonic fluorochrome solution containing 50 mg/ml propidium iodide (P.I.; Sigma Chemical Co., St. Louis, MO), 1% sodium citrate and 0.1% Triton X-100 (Sigma). Cells exhibiting DNA fragmentation and apoptosis were shown to be those in which P.I. fluorescence was weaker than the typical G_0/G_1 cell cycle peak. For analysis of cell morphology by flow cytometry, cells were resuspended in PBS containing 10% FCS.

DNA gels: Cells (10^6) were washed and resuspended in cold 10 mM Tris/ 1 mM EDTA (TE) buffer containing 0.2% Triton X-100. Debris was pelleted and supernatant transferred to a fresh tube. After addition of 35 ml of 3 M sodium acetate, DNA was extracted with phenol-chloroform. Fragmented DNA in supernatants was precipitated with ethanol, pelleted, rinsed with cold ethanol, dried, and resuspended in Tris/EDTA buffer. For gel electrophoresis, samples were added to loading buffer consisting of 40% sucrose in Tris/EDTA buffer, 1% SDS (Sigma), bromophenol blue, and 2.5 mg/ml RNase (Gibco/BRL), 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 volts for 2 hr and stained with ethidium bromide.

Immunohistochemistry: Freshly isolated bone marrow cells or CD45/B220$^+$ cells were cytospun onto glass microscope slides, air dried, and fixed in 10% formalin. Slides were then incubated with 2 mg/ml rabbit polyclonal anti-AhR antibodies or control rabbit immunoglobulin for 1 hour at 37° C, washed and incubated with a 1:300 dilution of biotinylated swine anti-rabbit immunoglobulin antibody for 30 min at 37° C. Background peroxidase activity was quenched with 3% H_2O_2 for 10 min, cells were washed, and horseradish peroxidase-conjugated streptavidin was added for a 25 min incubation at 37° C. AhR-specific staining was visualized by incubating slides in 0.1% 3,3'-diaminobenzidine and 0.04% H_2O_2 (DAKO, Carpinteria, CA) in PBS solution for 10 minutes at room temperature. Slides were then washed in running tap water and counter-stained with hematoxylin, coverslipped with Permount and examined by light microscope. AhR staining under these conditions was completely inhibited by absorbing anti-AhR antibody with sepharose beads conjugated with recombinant AhR protein. The stain was not affected by absorbing anti-AhR antibody with sepharose beads conjugated with an irrelevant protein, lysozyme (data not shown).

Southern blot analysis of Ig heavy chain gene rearrangements: Procedures for Southern blotting and hybridization using the JH probe "P2" have been previously described in detail (Near and Haber, 1989) with the exceptions that charged nylon filters

were used and that the DNA transfer was done using alkaline transfer as described by the manufacturer (Gene Screen Plus, NEN Research Products, Boston, MA).

RT-PCR for AhR mRNA: PreB cells were washed off stromal cell monolayers by vigorous pipetting and RNA prepared from 5 x 10^6 cells as described (Leedo Medical Laboratories, Houston, TX). Adherent stromal cells (3-5 x 10^6) were lysed directly in tissue culture wells. Integrity of RNA samples was assayed by electrophoresis in 1.5% agarose gels prior to RT-PCR to detect AhR mRNA in 5 mg samples (total RNA) as described (SuperScript Preamplification System; Gibco/BRL). cDNA was amplified for 35 cycles with $MgCl_2$ concentration adjusted to 2.5 mM to maximize specific signal and using the following AhR primers: CTGGCAATGAATTTCCAAGGGAGG and CTTTCTCCAGTCTTAATCATGCG. Primers were chosen to enclose the sequence which contains the putative murine AhR ligand-binding domain (Dolwick *et al.* 1993; Ema *et al.* 1992). Amplified DNA was electrophoresed through 3% gels (3:1 NuSieve:LE agarose; FMC, Rockland, ME) and DNA visualized by ethidium bromide staining. All samples were normalized for equal loading of gels with the GADPH housekeeping gene. GADPH mRNA was reverse transcribed and cDNA amplified with the following primers: CCATCACCATCTTCCAGGAG and CCTGCTTCACCACCTTCTTG.

In situ hybridization: ^{35}S Radiolabelled AhR riboprobes were generated using T7 (sense) and SP6 (anti-sense) promoters with linearized *XbaI* and *HindIII* digests of pcDNA-AhR murine AhR cDNA as template. PreB cells harvested from 4 week old bone marrow cultures were fixed with 4% paraformaldehyde, dehydrated and hybridized for 18 hr. at 52° C with either sense or anti-sense riboprobe. Slides were washed and auto-radiographs exposed for 6 weeks. No signal was observed with the sense riboprobe.

Terminal deoxynucleotide transferase (TdT) labeling for in situ analysis of apoptosis: In situ analysis of DNA integrity was assessed using the protocol of Tilly *et al* (Tilly *et al.* 1995). Bone was fixed and decalcified (Surgipath, Richmond, IL) for two days, changing the solution each day. Tissue was rinsed in water for 1 hr, embedded in paraffin, sectioned, and mounted unstained onto microscope slides. Paraffin was removed by incubating 30 min in a 60° C oven followed by a 10 min wash in xylenes. Tissue was rehydrated with graded concentrations of ethanol as follows: 100% ethanol for 10 minutes, 90% ethanol for 3 min, 2% H_2O_2 in 90% methanol for 1 min, 80% ethanol for 3 min, 70% ethanol for 3 min, and sterile water for 3 min. Each section was treated with proteinase K (10 mg/ml in 2 mM $CaCl_2$, 20 mM Tris-HCl, pH 7.4) and incubated for 30 min at 37° C. Slides were washed twice with sterile water. Tissue was pre-equilibrated with 5X TdT reaction buffer provided by the manufacturer and $CoCl_2$ (Boehringer Mannheim, Indianapolis, IN) for 20 min. Slides were then incubated for 15 min at 37° C with the reaction mixture for 3'-end labeling. The reaction mixture consisted of TdT reaction buffer, 5 mM $CoCl_2$, 50 mM biotin-16-dUTP, 500 mM dUTP, and 25 units/0.1 ml TdT enzyme (Boehringer-Mannheim). Slides were washed 3 times for 10 min each in 150 mM NaCl, 100 mM Tris-HCl (pH 7.5) buffer and then blocked by incubation with 3% BSA (w:v) in buffer for 30 min at room temperature. ABC reagent (Vector Labs, Inc. Burlingame, CA) was added, slides were incubated at room temperature for 15 min, dipped in buffer (100 mM NaCl, 100 mM Tris-HCl, pH 9.5, and 50 mM $MgCl_2$) and washed 3 times for 10 min each in buffer. DAB (Vector Labs) was used to detect

localization of incorporated biotin-dUTP in apoptotic cells. Reactions were stopped with TE buffer and slides counterstained with hematoxylin, followed by a lithium carbonate wash. Sections were dehydrated with a graded ethanol series (70% - 100% ethanol; 1 min each) and allowed to air dry before coverslip mounting with Permount).

Transient transfections: Exponentially growing cultures of 3T3 cells were transfected with 10 mg of the appropriate plasmid DNA plus 20 mg of salmon sperm carrier DNA by calcium phosphate co-precipitation. After 12 hours, the transfection medium was removed and replaced with fresh culture medium. 12 hours later the cells were placed in culture medium containing 10% or 0.5% serum. 18 hours later the transfected monolayers were washed with PBS and cytosolic protein was obtained as described above. Cell extracts were assayed for luciferase activity using a commercially available kit (Promega).

RESULTS.

Activation of the Aryl Hydrocarbon Receptor/Transcription Factor Induces NF-κB/Rel.

Benzo[α]pyrene induces NF-κB in mouse hepatoma cells: PAH ability to activate NF-κB in hepatoma cells, Hepa-1, was determined by treating the cells for 1 hour with vehicle or with 10^{-5} to 10^{-8} M B[α]P. Following the treatment, cell nuclei were isolated and nuclear protein was evaluated by EMSA for NF-κB oligonucleotide binding. The nuclear fraction from vehicle-treated or untreated Hepa-1 cells demonstrated a minor background level of constitutive NF-κB binding. B[α]P treatment significantly increased the NF-κB band on the EMSA gel. The same molecular weight band was increased on the EMSA gel separating nuclear protein from the cells treated with 10^{-9} M PMA, a potent NF-κB inducer. These results suggest that in Hepa-1 cells B[α]P induces NF-κB binding to the Igκ light chain gene promoter containing the core NF-κB binding site.

These data require confirmation that NF-κB oligonucleotide binding is specific. In order to test specificity, EMSA was performed with the nuclear protein isolated from 10^{-6} M B[α]P treated Hepa-1 cells in the absence or presence of 100 fold excess of unlabelled NF-κB binding oligonucleotide or an irrelevant oligonucleotide (XRE). The NF-κB band induced by B[α]P treatment disappeared in the samples substituted with an excess of cold NF-κB probe, but not with irrelevant XRE oligonucleotide. It proves that B[α]P induced EMSA signal represents a member of the NF-κB/Rel family.

7,12 Dimethylbenz[a]anthracene induces NF-κB in mouse hepatoma cells: It would be of interest whether ability to activate NF-κB is restricted just to B[α]P or if it might be common for different PAHs. To answer this question Hepa-1 cells were treated for 1 hour with vehicle or with 10^{-5} and 10^{-6} M DMBA, another carcinogenic (RamaKrishna *et al.*, 1992) and immunosuppressive (Thurmond *et al.*, 1988) PAH/AhR ligand. NF-κB binding activity of nuclear proteins was evaluated by EMSA. DMBA induced NF-κB binding at the tested concentrations. These data suggest that NF-κB activation in mouse hepatoma cells may be generalized to other AhR-binding PAH.

Role of the AhR in B[α]P-induced NF-κB activation: It could be hypothesized by analogy with other PAH effects that B[α]P-induced NF-κB activation is an AhR-dependent process. To examine this hypothesis, we tested if NF-κB activation induced by B[α]P would be prevented by a known inhibitor of AhR activity, α-NF (Gaseiwicz and Rucci, 1991; Kawabata and White Jr., 1987). α-NF competitively binds the AhR, prevents AhR nuclear translocation, and inhibits AhR-regulated cytochrome P-4501A1 activity (Wilhemsson *et al.*, 1994; Blank *et al.*, 1987; Merchant, Arellano, and Safe, 1990). One to two hours treatment of Hepa-1 cells with 10^{-5}-10^{-6} M α-NF alone did not activate NF-κB. 1 hour cell pretreatment with 10^{-6} or 5 x 10^{-6} M α-NF completely prevented the B[α]P-induced NF-κB activation. These data support that the AhR and/or AhR-controlled P-450 enzymes play a role in B[α]P-induced NF-κB activation.

Another way to test if activation of the AhR is a necessary part of NF-κB activation by PAH would be to use B[*e*]P. B[*e*]P is a B[α]P stereoisomer which has low affinity to the AhR and is proportionately less bioactive (White Jr and Holsapple, 1984;

Wojdani and Alfred, 1984; White Jr., Lysy, and M.P., 1985; Yamaguchi *et al.*, 1997). If the AhR mediates the process, B[*e*]P would not effect NF-κB activation. As predicted, treatment of Hepa-1 cells with 10^{-5} to 10^{-7} M B[*e*]P did not increase NF-κB activity, although, exposure to B[α]P elevated NF-κB binding. This is also consistent with a role for the AhR in NF-κB induction.

To further investigate the suggested role of the AhR in B[α]P-induced NF-κB activation, Hepa-1 cells and mutants deficient in the AhR machinery were tested for their ability to induce NF-κB after B[α]P treatment. Hepa-1 mutant, BP^{r}C1, has a normal level of the AhR protein but is defective in expression of ARNT. Ligated AhR in BP^{r}C1 cells does not translocate to the nucleus (Miller, Israel, and Whitlock Jr., 1983), and these cells do not induce cytochrome P-4501A1 in response to PAH (Near *et al*, submitted). Another Hepa-1 mutant lacking the AhR protein is Hepa-1C2 (Hankinson, 1994). Unfortunately, one can not rule out the possibility that there is more than one mutation in the hepatoma sublines, so AhR functioning is not the only difference. All three cell lines, Hepa-1, BP^{r}C1 and Hepa-1C2, are capable of NF-κB activation in response to inducers like PMA and/or H_2O_2. However unlike Hepa-1 cells, BP^{r}C1 and Hepa-1C2 do not increase NF-κB binding consequently to B[α]P treatment. These results provide indirect evidence that functional a AhR complex is required for B[α]P induced NF-κB activation in murine hepatoma cells.

Cytochrome P-4501A1 activity is not required for benzo[α]pyrene-induced NF-κB activation: The most extensively studied case of a gene expression induced by AhR binding to promoter XRE is expression of cytochrome P-4501A1 (Nebert, Petersen, and Fornace Jr., 1990; Lusska, Shen, and Whitlock Jr., 1993). Some baseline level of P-450 1A1 activity is observed constitutively (Schmidt *et al.*, 1996). This background amount of P-450 could be enough to metabolize PAH in the cells where AhR activation is impaired, so these cells as well could generate some small quantities of reactive PAH intermediates. In this context, B[α]P induced NF-κB activation could be inhibited by α-NF either due to inhibition of the AhR or due to cytochrome P-450 inhibition. To find which of two options is really the case, and to test if cytochrome P-450 1A1 is required for NF-κB induction by B[α]P, we used a hepatoma cell line, Hepa-1C37 (Hankinson *et al.*, 1985), defective in expression of the P-450 encoded by *CYP1A1* gene due to missense mutations. The ethoxyresorufin deethylase (EROD) assay reveals that Hepa-1C37 is incapable of inducing P-4501A1 enzyme activity in response to treatment with AhR ligands, including the high affinity AhR ligand, TCDD, although, this cell line expresses normal level of the AhR protein. This defect does not prevent B[α]P from induction of NF-κB in Hepa-1C37 cells. The same B[α]P concentration simultaneously used in control Hepa-1 cells resulted in NF-κB induction. These results demonstrate that cytochrome P-450 1A1 activity is not required for NF-κB activation by B[α]P.

Expression of the Aryl-Hydrocarbon Receptor is Regulated by Serum and Mitogenic Growth Factors in Murine 3T3 Fibroblasts.

Growth arrest is associated with decrease or lack of AhR expression: Our first goal was to test whether growth arrest of Swiss 3T3 cells parallels in time with a change

of intracellular AhR level. Swiss 3T3 fibroblasts had been grown exponentially in the medium substituted with 10% serum. To growth-arrest the cells they were put in medium containing 0.5% serum for 24 hours. The degree of growth arrest was confirmed by FACS scan analysis of propidium iodide-stained nuclei. The growth-arrested cell population had fewer cells in S and G_2+M phases of a cell cycle comparing with cells constantly grown in a serum rich media. Cytosolic protein obtained from exponentially growing and growth-arrested fibroblasts were tested by Western blotting analysis with anti-AhR antibodies. The analysis revealed a dramatic decrease of AhR protein level in the serum-starved cells.

To test if the reduction of AhR level induced by growth arrest has functional implications, exponentially growing and growth arrested fibroblasts were treated with B[α]P for 45 minutes, and their nuclear proteins were tested for XRE-binding activity by EMSA. Normalized amounts of nuclear protein from serum-starved fibroblasts revealed lower XRE-binding activity following B[α]P-stimulation than the nuclear protein from proliferating cells. This result demonstrates that AhR functioning, including response to PAH, is impaired in the growth arrested cells which posses reduced level of the AhR protein.

To test if the reduction of the AhR level induced by growth arrest is reversible, serum-starved cells were put back to the 10% serum containing medium and retested for cellular AhR protein by Western Blot after indicated periods of time (so the kinetics of the AhR induction were obtained). Immunoblot analysis of the AhR revealed a lag period of 13 hours following addition of serum before AhR is detectable. AhR protein level restores completely after 18 hours of stimulation with serum. Cell cycle kinetics were based on the time course of [^{3}H]-thymidine incorporation into genomic DNA. Our experiments demonstrate that DNA synthesis begins about 13 hr after serum-stimulation of quiescent cells, and is completed after 22-24 hours. AhR expression was detected at the G_1/S-phase boundary, and peaked in the late S-phase.

Mechanism of AhR induction by serum: To study if the growth arrest effects AhR transcription, we used transiently transfected Swiss 3T3 cells with the reporter p5K600f plasmid containing luciferase gene controlled by AhR promoter (Schmidt *et al.*, 1993). The transfectants were either grown exponentially in 10% serum or growth arrested by serum deprivation (0.5% serum) for 18 hours, harvested, and their cytosolic proteins were tested for luciferase activity which represents the transcription from the AhR promoter. This experiment demonstrated that growth arrest reduces the luciferase activity in p5K600f-transfected cells by almost 90% versus transfectants constantly maintained in 10% serum. This reduction could not result from nonspecific overall repression of gene transcription, because growth arrest did not effect luciferase activity in Swiss 3T3 cells transiently transfected with a promoterless luciferase construct (pGL2b) or a vector containing the luciferase gene downstream of a strong constitutive promoter (pSV2LUC). These data suggest that reduction of the AhR protein level induced by growth arrest is regulated by transcription.

Specific growth factors induce AhR expression in Swiss 3T3 cells: To determine which components of a serum are responsible for induction of AhR expression, e.g. defined fibroblast mitogens or something else, growth arrested fibroblasts were

stimulated with serum, Platelet-Derived Growth Factor (PDGF), and basic Fibroblast Growth Factor (bFGF), and tested for the AhR level by Western Blotting and for cell cycle status by measurements of [^{3}H] thymidine incorporation. [^{3}H]-thymidine incorporation in serum-starved 3T3 cells following the treatment demonstrated that PDGF and bFGF rescued cells from growth arrest. Among all three stimuli serum was the strongest inducer of AhR expression, but PDGF and bFGF induced the AhR significantly as well. It was demonstrated by immunoblot analysis that the nuclear fraction of the serum or mitogen stimulated fibroblasts also contained increased level of the AhR compared to the nucleus of growth arrested cells. These data suggest that it mitogens in serum induce AhR expression and regulate the amount of AhR present in the nuclear compartment during the cell cycle.

Cell cycle progression is dissociable from AhR induction: The experiments described above demonstrate a correlation between cell rescue from the growth arrest and induction of AhR expression. Nevertheless, these experiments do not reveal whether the progression through the entire cell cycle is necessary for AhR induction. To address this question, we tested whether sodium-butyrate, a cell cycle inhibitor, prevents AhR expression concurrently with blocking of serum/ mitogens induced cell cycling. Growth arrested fibroblasts were put in 10% serum media in the presence or absence of 2 mM sodium-butyrate, and tested for entry into the cell cycle by [^{3}H]-thymidine incorporation assay, or for AhR expression by Western blot. Although this non-toxic dose of sodium-butyrate prevented serum-stimulated DNA synthesis (blocking the cells in G_1) it did not affect serum-dependent induction of AhR expression. This demonstrates that progression of the cell through the entire cycle is not necessary for serum-dependent induction of AhR expression in Swiss 3T3 fibroblasts.

Activation of the Aryl Hydrocarbon Receptor/Transcription Factor and Bone Marrow Stromal Cell-Dependent PreB Cell Apoptosis.

DMBA induces apoptosis in a preB cell line: To determine the stage of B cell development represented by our model cell line, BU-11, we tested BU-11 cells by flow cytometric analyses for expression of CD43, B220/CD45 and IgM surface antigens, and by southern blotting analysis for immunoglobulin heavy chain gene rearrangement. The analysis reveals that BU-11 cells are CD43 and B220/CD45 positive but lack surface IgM, a phenotype of primary pro B cells (Hardy *et al.*, 1991). We refer to BU-11 as early preB cells because, according to Southern blotting, their heavy chain genes are rearranged. Formally they have to be nomenclatured as B cells at the transition point between pro- and preB cells.

To study the toxic outcomes of bone marrow stromal/preB cell cultures exposure to DMBA, BU-11 cells growing on BMS2 stromal cells were treated with 10^{-4} M DMBA, harvested 18 hours after the treatment, and evaluated for percentage of apoptosis by flow cytometric analyses of cell morphology and by DNA staining with propidium iodide (Herdin *et al.*, 1992). Only 2% of vehicle treated cells were apoptosing according to a characteristic dull P.I. staining pattern, and only 6% were characterized as apoptotic by morphologic features like reduced size (lower forward scatter on FACS) and increased

granularity (increased side scatter). Condensation of the cells treated with DMBA and change of their nuclei morphology were visible by light microscopy. Both DNA staining and morphologic assays demonstrated an increase in apoptosis up to approximately 35% consequent to DMBA exposure. Similar results were obtained with BU-11 subclones and with primary preB cells from Witlock/Witte (Whitlock *et al.*, 1984) cultures.

Another method of determining apoptosis is a demonstration of apoptosis specific DNA fragmentation by 3.5% agarose gel electrophoresis. This technique was used to test DNA extracted from BU-11 cells treated with 10^{-4} M DMBA for 12, 18 or 24 hours. DNA digestion into oligonucleosomal fragments represented on the gel by a typical ladder pattern occurred after 12 hours of exposure to 10^{-4}-10^{-6} M DMBA or after 24 hours treatment with 10^{-8} M DMBA ($p \leq 0.01$). The results of these experiments were not sensitive to possible contamination with supporting BMS2 stroma cells, because DMBA did not induce DNA digestion in these cells.

To summarize these results, DMBA induced pre B cell apoptosis is a possible mechanism of PAH immunotoxicity. The apoptosis is induced after short treatment with low doses of DMBA similar to the doses of dexamethasone inducing apoptosis in thymocytes. Similar results were obtained using long-term primary murine bone marrow cultures as a model to test DMBA induced apoptosis of early preB cells. Long-term primary murine bone marrow cultures is a model closer to *in vivo* situation emphasizing the physiological relevance of these results.

DMBA -mediated apoptosis is blocked by α-napthoflavone, an AhR and cytochrome P450 antagonist: To determine if DMBA induced apoptosis of pre B lymphocyte is mediated by the AhR and/or enzymes controlled by the AhR (i.e. P-450 1A1 and 1A2), long-term primary murine bone marrow cultures or BU-11 lymphocytes grown on BMS2 cells were treated with vehicle or with 10^{-4}-10^{-6} M DMBA in the presence or absence of 10^{-6} M α-NF. At this concentration, α-NF alone did not induce BU-11 cells apoptosis. Instead, it prevented apoptosis otherwise induced by 10^{-5} or 10^{-6} M DMBA, and reduced apoptosis induced with 10^{-4} M DMBA. These results suggest a role for the AhR and/or P-450 1A1 as mediators of DMBA induced pre B lymphocyte apoptosis.

BMS2 but not preB cells express high levels of AhR protein and mRNA: If the AhR mediates DMBA induced apoptosis of BU-11 cells grown on top of supporting BMS2 stroma layer, one can ask which of two interacting cell lines possesses the AhR. To answer this question, we tested both cell lines for presence of AhR protein and mRNA.

Western blotting with monoclonal and polyclonal anti-AhR antibodies detected AhR protein in BMS2 whole cells protein fraction but not in BU-11 cells separated from BMS2 cells. To assess the ability PAH to functionally activate the AhR in BMS2 cells, the cells were treated with vehicle, DMBA or B[α]P, and their nuclear protein was tested for the level of the AhR by immunoblotting. AhR nuclear translocation in Hepa-1 cells induced by TCDD was used as a positive control. This experiment demonstrate that the AhR in BMS2 cells is capable of nuclear translocation in response to PAH treatment. The AhR machinery is functional in BMS2 cells but does not exist in BU-11.

AhR mRNA was found in BMS2 but not in BU-11 cells by *in situ* hybridization of BU-11/BMS2 cultures with an AhR antisense riboprobe. There was no signal in either cell line when *in situ* hybridization was performed with an AhR sense riboprobe as a specificity control.

Another technology used to reveal AhR mRNA distribution was AhR mRNA-specific RT-PCR. Given the extremely high sensitivity of this technique, one could predict that even minor contamination of BU-11 cells with BMS2 could give false-positive results. In order to avoid this, BU-11 cells were cultured for 7 days in rIL-7, a cytokine sufficient to support lymphocyte growth. It could be that this change of the BU-11 supporting environment from BMS2 cells to rIL-7 cytokine changed AhR expression , but we do not think it accounts for the result given similar observations obtained by other techniques. No AhR mRNA was found in rIL-7 supported BU-11 cells. BMS2 cells were highly AhR mRNA positive. Quantitative RT-PCR with a competitive AhR mimic indicated that BMS2 cell has about 25% of the amount of AhR mRNA found in Hepa-1 cells (2700 molecules/cell in BMS2 vs. 10,600 molecules/cell in Hepa-1). It is remarkable that Hepa-1 is highly AhR expressing hepatoma line which has been used as a standard (Cuthill, S. *et al.*, 1987).

The presence of AhR protein and mRNA in supporting stroma cells and the lack of them in supported pre B lymphocytes was also shown in long-term primary preB cells cultures.

BMS2 cells are required for B cell apoptosis: The fact that the AhR is present only in supporting stroma cells, and the notion that DMBA induces pre B cell apoptosis via the AhR raised the hypothesis that DMBA would not induce BU-11 cell apoptosis in the absence of BMS2 cells. To test this hypothesis, BU-11 cells were passed 3-4 times in rIL-7 during 10-14 days, so they could be considered BMS2 free. Lymphocytes were exposed for 24 hours to 10^{-6}-10^{-8} M DMBA and apoptosis was quantitated. In agreement with the original prediction, rIL-7 supported BU-11 did not respond with apoptosis to DMBA, although 10^{-6} M is a dose two orders of magnitude higher than the minimal dose inducing apoptosis in BMS2 supported BU-11 cells.

To test if rIL-7 supported BU-11 cell loss of ability to apoptose in response to DMBA is reversible, BU-11 cells supported by rIL-7 were transferred back on BMS2 monolayers and treated with DMBA in the presence of rIL-7. BMS2 support restored capability of BU-11 cells to apoptose after DMBA treatment. The presence of rIL-7 did not interfere with apoptosis. Hence the cytokine per se does not make lymphocytes incapable of apoptosis or DMBA resistant.

We think that exposure to DMBA stimulates the functional ability of BMS2 to support BU-11 apoptosis, e.g. by BMS2 production of factors(s) stimulating apoptosis. Similar experiments and results with polyclonal long-term primary preB cells demonstrate that this is unlikely to be a clonal artifact.

Fluoranthene-induced Apoptosis in Murine T Cell Hybridomas IsIndependent of the Aromatic Hydrocarbon Receptor.

Fluoranthene induces apoptosis in T hybridoma cells. It is important to know if non-halogenated PAH, and fluoranthene in particular, compromise T cell branch of immune response, and if apoptosis is a mechanism of PAH immunotoxicity for T lymphocytes like it is for B cells. ME1 T hybridoma cells were used as a model. These cells were treated with vehicle or with 0.2 mM fluoranthene, harvested, and tested for apoptosis by DNA staining with propidium iodide and flow cytometric analyses of cell morphology (Hardin *et al.,* 1992), like it was in case with DMBA treated pre B cells. Both techniques characterized only 7% of vehicle-treated cells as apoptotic ones (i.e. smaller and more granular cells with decreased P.I. staining). The same techniques revealed an increase in apoptosis up to 36-39% consequent to 12 hours treatment with fluoranthene. Assessment at 18 to 48 hours showed no increase in percent of apoptosis over 12 hours. ME1 T hybridoma apoptosis was also observed 24 - 48 hours after treatment with only 0.1 mM fluoranthene. Overall, these experiments may be interpreted as ability of fluoranthene to induce apoptosis of T hybridoma cells.

As it was described above for evaluation of pre B apoptosis, assay for DNA fragmentation typical for apoptosis were performed on ME1 cells treated with vehicle, fluoranthene for 4, 8, 12, or 18 hours, or dexamethasone for 12 hours as a positive control. Extracted DNA was electrophoresed in 3.5% agarose gels. DNA isolated from vehicle-treated cells or cells treated with fluoranthene for 4 hours demonstrated some background level of DNA digestion into fragments of oligonucleosome size. 8 hours of treatment with fluoranthene significantly increased DNA digestion which peaked at 12 hours.

Fluoranthene-induced apoptosis in additional T cell hybridomas. Fluoranthene - induced apoptosis could be a phenomenon general for T cell hybridomas or a clonal artifact of the ME1 cell line. In order to test which of two possibilities is correct, three additional hybridomas 5D5, K31H28, and 12.13 were tested for apoptosis due to fluoranthene treatment. Lines K31H28, and 12.13 were as apoptotically responsive to fluoranthene as ME1. Hybridoma 5D5 differed from other tested lines and did not respond apoptotically. These data demonstrated that fluoranthene-induced apoptosis is a common feature for a number, but not all T cell hybridomas, and raises the question of which characteristics T cells must possess to be sensitive to fluoranthene.

Fluoranthene-induced T cell hybridoma apoptosis is not Ah receptor-dependent: It is commonly thought that PAHs induce immunotoxicity via AhR activation (White *et al.,* 1985; Morris *et al.,* 1994; Hardin *et al.,* 1992; Silkworth and Vecchi, 1985). A possible role for the AhR has to be studied in each particular case (i.e. type of PAH and type of cell) because most lymphocyte subsets were not characterized as to whether they have the AhR or not. Neither, fluoranthene was not adequately tested to see if it functionally activates the AhR, although this was assumed from its chemical structure. Two strategies were used to reveal if the AhR mediates fluoranthene-induced apoptosis of T hybridomas. First, fluoranthene was tested for it's potential to induce AhR nuclear translocation. Second, levels of the AhR protein and mRNA were mesured in T hybridoma cells .

As described above, the Hepa-1 cell line is a model for AhR expression and activation (Singh and Perdew, 1993). Fluoranthene was tested for it's ability to activate AhR nuclear translocation by Western blot of nuclear protein. B[α]P and DMBA were used as a positive control demonstrating induction of AhR nuclear translocation proportional to their affinities for AhR (Bigelow and Nebert, 1982). In contrast, 0.2 mM fluoranthene did not initiate any detectable AhR nuclear translocation in Hepa-1 cells, although this concentration is high enough to induce ME1 apoptosis. These experiments did not rule out a possibility of such fluoranthene-induced AhR-mediated second messenger signaling as protein phosphorylation (Enan and Matsumura, 1995), which was not shown to require AhR nuclear translocation.

Another fact suggesting that the fluoranthene-induced apoptosis is not mediated by the AhR is lack of AhR protein on Western blots of ME1, K3lH28, or 12.13 protein fractions. Series dilutions of Hepa-1 proteins tested by AhR immunoblotting showed the specific band even at the amount of AhR protein approximately equal to 500 pg. The lack of AhR protein in ME1, K3IH28, and 12.13 cells correlated with absence of AhR mRNA in these cells demonstrated by RT-PCR. Hepa-1 mRNA was used as a positive control demonstrating sensitivity of RT-PCR assay as 100-200 mRNA copies. Concluding these results, the lack of AhR protein and mRNA in T hybridoma cells, and the inability of fluoranthene to activate the AhR suggest that fluoranthene-induced T cell apoptosis is not Ah receptor-dependent.

Fluoranthene-induced apoptosis is partially ion-dependent. Ca^{2+} is a signaling molecule controlling a multitude of biological responses including apoptosis (Nicotera *et al.*, 1989). PAH induce increase of intracellular Ca^{2+} concentration (Burchiel *et al.*, 1992), leading to lymphocyte apoptosis and disruption of cell functioning (Davila *et al.*, 1995; Karras and Holsapple, 1994). To test if fluoranthene induces extracellular ion flux involved in T hybridoma apoptosis, the ion chelator, EGTA, was added to cultured ME1 cells with or without fluoranthene for 16 to 24 hours. After the incubation, ME1 cells were harvested and tested for apoptosis. 1 to 2 mM EGTA did not increase background level of ME1 apoptosis if EGTA was administered by itself, but significantly reduced fluoranthene-induced apoptosis. These data suggest the role of Ca^{2+} signaling in fluoranthene-induced apoptosis of ME1 cells.

DISCUSSION.

Within the last couple of decades, epidemiologists, toxicologists and molecular biologists have paid increased attention to the adverse effects of PAH and their molecular mechanisms. Multiple research brought up a dominating point of view that the AhR mediates toxic effects of PAH, and a lot of information was gained within this paradigm. Nevertheless, several inter-related questions remain to be addressed. From the mechanistic point of view, AhR interaction with other intracellular signaling molecules was not studied in depth. Many of signaling messengers are tightly controlled by a cell cycle and in order to interact with the AhR they have to be present in the cell simultaneously. Expression of the AhR is poorly studied, and it was not revealed whether AhR expression is controlled by the cell cycle and/or mitogens. From a toxicological point of view, it is of interest if PAH is toxic for developing B lymphocytes, T cells, and what could be the mechanism of such PAH toxicity. In the present work, we focused on these mutually complementing questions.

Induction of NF-κB/Rel is Mediated by Activation of the Aryl Hydrocarbon Receptor/Transcription Factor.

NF-κB modulation by PAH and/or activation of the aryl hydrocarbon receptor/transcription factor was studied in the present research. A Hepa-1 mouse hepatoma cell line treated with B[α]P or DMBA served as a model to demonstrate that PAH treatment induces a protein or protein complex in the nuclear fraction which binds an oligonucleotide containing the NF-κB binding site from the Igκ light chain promotor. This binding was revealed as increased activity of a shifted band on the EMSA gels. There are two reasons to believe that activated protein or protein complex was NF-κB. First, the effect was specific: the B[α]P induced EMSA band was prevented by excess of unlabelled oligonucleotide containing the NF-κB binding site, but not by a nonspecific oligonucleotide (XRE sequence). Second, a known NF-κB activator, PMA, induced the gel shift band of the same mobility as did B[α]P.

If PAH activate NF-κB, than AhR ligands may influence expression of multiple NF-κB-controlled genes which do not have XRE in their promoters. For example, this may be the reason why PAH induce replication of viruses containing NF-κB in the regulatory elements (Nabel and Baltimore, 1990; Thrower *et al.*, 1996). Epidemiologically, such virus stimulation was inferred by increased AIDS progression in smokers (Neiman *et al.*, 1993). Although the HIV viral genome contains an XRE, it was shown (Yao *et al.*, 1995) that TCDD-induced transcription of a chloramphenicol acetyltransferase (CAT) reporter gene construct controlled by the HIV-LTR is due to its NF-κB binding site, at least to some extent. The same system (Yao *et al.*, 1995) was used to demonstrate B[α]P-induced transcription of the HIV-LTR-driven reporter construct, but at the PAH concentrations three orders of magnitude higher than the minimal dose (10^{-8} M) active in our system. NF-κB activation may explain PAH-induced modulation of cytokine production (Moos, Oughton, and Kerkvliet, 1997), and PAH immunosuppression which may be controlled by the cytokines (Holsapple *et al.*, 1996).

The future *in vivo* experiments will have to address physiological consequences of long term exposure to low PAH doses.

There are three types of experiments suggesting that either the AhR or AhR regulated genes mediate B[α]P/PAH-induced NF-κB activation. First, α-NF- which blocks AhR nuclear translocation (Gaseiwicz and Rucci, 1991)- and P-450 1A1 activity (Gurtoo *et al.*, 1979) prevents NF-κB induction by treatment with B[α]P. Second, a B[α]P stereoisomer, B[*e*]P, fails to activate both NF-κB and the AhR. A third line of evidence is obtained by Hepa-1 mutants impaired either in AhR functioning (BP^{r}C1) or AhR expression (Hepa-1C2). All these cell lines have NF-κB which may be activated by PMA and/or H_2O_2. Nevertheless, BP^{r}C1 and Hepa-1C2 mutants fail to activate NF-κB in response to B[α]P treatment.

If PAH induces NF-κB via AhR activation, does oxidative stress, a known mechanism of NF-κB induction (Schreck, Albermann, and Baeuerle, 1992), play a role? AhR-induced cytochrome P-450 oxidizes PAH to the oxidative stress inducing intermediates. In turn, oxidative stress due to these intermediates activates NF-κB). Although both B[α]P and DMBA are highly metabolizable, the activity of P-450 1A1 is unlikely to be required for PAH induced NF-κB activation, because B[α]P did up-regulate NF-κB in P-4501A1 defective Hepa-1 mutant, Hepa-1C37. This does not by itself rule out involvement of other genes induced by AhR, e.g., CYP 1A2, CYP 1b1. Do these metabolize DMBA or B[α]P?

While there are differences between our results and these of Yao *et al.* (Yao *et al.*, 1995), we do not think they contradict each other. In Yao's experiments, TCDD failed to induce the HIV-LTR-controlled reporter gene transcription when the construct was transfected to Hepa-1C37 cells. First, we used different NF-κB binding sites, HIV-LTR vs. Igk light chain promotor regions. These systems have differential responses. For example, DMBA did not stimulate induction of HIV-LTR-CAT activity (Yao *et al.*, 1995), but induced NF-κB in our system. Second, it is not known whether TCDD induces the same NF-κB/Rel subunits (Yao *et al.*, 1995) as PAH induced in our system. Last, our research focused on early events (1 hour after the treatment). Yao's group measured reporter gene transcription 24 hours after the treatment, which could be due to mechanism secondary to the exposure.

PAH induced NF-κB activation may be paralleled with B[α]P or DMBA-induced apoptosis of pre-B lymphocytes growing on bone marrow stromal cells (Yamaguchi *et al.*, 1997; Hardin, Hinoshita, and Sherr, 1992) or Hepa-1 monolayers (R. Near *et al*, submitted). Both effects of PAH require the AhR (in case of apoptosis, the AhR has to be present in lymphocyte supporting cells) but do not require P-450 1A1. The studies discussed in this chapter and "Activation of the Aryl Hydrocarbon Receptor/Transcription Factor and Bone Marrow Stromal Cell-Dependent PreB Cell Apoptosis" section will be continued by testing whether AhR-dependent NF-κB activation participates in apoptosis of immature lymphocyte induced by PAH.

Serum and Mitogenic Growth Factors Regulate Expression of the Aryl-Hydrocarbon Receptor in Murine 3T3 Fibroblasts.

The PAH induced NF-κB activation described above adds to the growing body of evidence that ligated AhR exhibits cross-talk with other signaling pathways. However, a search for AhR modulated intracellular signals will be inefficient and will depend on a random luck until we know how AhR expression is controlled and at which stages of cell cycle the AhR is expressed. In this part of our research, we have shown that serum and/or mitogens control AhR expression in 3T3 fibroblasts. This observation may be indirect evidence for an AhR role in cell division, analogous to other serum/mitogen induced genes (e.g. *c-myc, C-fos, c-jun, egr-1)* known to be involved in cell growth and differentiation.

An analogy with these serum/mitogen induced genes may be supported by the fact that all of them including the gene coding for the AhR are expressed by purified PDGF and bFGF (Pardee, A.B., 1989). This implies that mitogens are the components of serum responsible for AhR expression. Exiting growth arrest is a necessary and sufficient step for serum-induced AhR expression. However, progression of the cell through an entire cycle is not needed, because sodium-butyrate treatment inhibited cells in G_1 but did not abolish AhR expression.

Serum-stimulated AhR expression is maximal after replication of DNA. Serum-stimulated genes induced in early G_1 without protein synthesis necessary for their expression are called "immediate early" genes. *C-fos* and *c-myc* are among these genes. Genes like ornithine decarboxylase are expressed in late G_1, and their expression depends on protein synthesis. These genes expressed before S phase are called "delayed-early" genes. Genes which have peak of their expression after the onset of DNA synthesis are defined as "late" genes (Pardee, A.B., 1989). According to this nomenclature, AhR is a "late" gene.

The kinetics of AhR expression is consistent with the structure of the AhR promoter which contains AP-1 and *c-myc* binding sites. AP-1 is a transcription factor formed by dimerisation of *c-fos* and *c-jun.* All three, *c-myc, c-fos*, and *c-jun*, are serum/mitogen-induced transcription factors expressed earlier in the cell cycle. Furthermore, *c-myc* expression is reduced in growth-arrested cells (Rollins, B.J., 1987), and, like it is with the AhR, treatment with serum and/or purified mitogens (PDGF and bFGF) restores *c-myc* level (Armelin, H.A., 1984). It could be that physiological regulation of a sequentially ordered transcriptional program is executed by serum stimulation of *c-myc, c-fos*, and *c-jun* as "early" genes providing transcription factors for AhR "late" gene.

The physiological ligand for the AhR is unknown (if it exists at all), but sensitization of the cell in G_1 phase to this putative AhR ligand could be a physiological function of serum/mitogen-induced AhR expression. The nuclear fraction of serum/mitogens stimulated 3T3 cells contains AhR protein, suggesting functional modulation (at least nuclear translocation) of the AhR by growth factors. Proliferating fibroblasts are also more responsive to AhR ligation by toxins than growth-arrested cells (e.g. B[α]P stimulates grater EMSA signal).

The toxic response of fibroblast to AhR ligation may be exemplified by inhibition of terminal adipocyte-conversion of 3T3-L1 fibroblasts (derived from Swiss 3T3 cells) consequent to TCDD exposure (Phillips, M., 1995). This could mean that the AhR triggers proliferation and differentiation. The AhR involvement in mouse development is also supported by the report that homozygous AhR "knockout" mice possesses developmental abnormalities. Both these facts suggest high significance for tight transcriptional control of AhR expression.

3T3 fibroblasts have been used as a model to study serum-induced factors involved in growth and differentiation common for many different cell types. Results of this research could also turn out to be of a general nature, but even if they are not, it is important by itself that AhR expression in fibroblasts is controlled by mitogens. It suggests a role for the AhR in such vital processes controlled by fibroblast proliferation as organogenesis, the maintenance of connective tissue, and wound healing.

Bone Marrow Stromal Cell-Dependent PreB Cell Apoptosis and Activation of the Aryl Hydrocarbon Receptor/Transcription Factor.

Apoptosis is a cell suicide process involved in multiple physiological functions and pathologic manifestations including formation and control of both T and B lymphocyte repertoires, and, as a result, autoimmunity and immunodeficiency. Any environmental factor interfering with the normal course of apoptosis is a potential disrupter of T and B cells development and immune response. PAH are among the environmental toxins inducing lymphocyte apoptosis, as was demonstrated with B[α]P and DMBA (Burchiel *et al.,* 1992, 1993; Hardin *et al,* 1992; Hinoshita *et al.,* 1992). PAH-induced lymphocyte apoptosis is likely to be one of the mechanisms of PAH immunotoxicity (e.g. suppression of macrophages, T and B cells), a phenomenon under scrutiny in the recent years (reviewed in Davila *et al.,* 1995) due to it's scientific and public health importance.

As discussed above, control of cell's developmental fate could be a physiological function of the AhR. The AhR could be involved in the "decision making process" choosing whether the cell should grow further, differentiate, or undergo programmed cell death/apoptosis. Our data obtained from the model of DMBA treated pre B cells/stroma cells support involvement of the AhR in apoptosis of maturing B lymphocytes. DMBA treated cells demonstrated dull P.I. staining, DNA digestion into oligonucleosome size fragments, and altered cell morphology characteristic of apoptosis. Such a clonally non-specific induction of developing lymphocyte apoptosis suggests that B cell branch of immune response will be overwhelmed by DMBA and other apoptosis inducing PAH, and these compounds will skew the final lymphocyte repertoire.

There are three lines of evidence in our research which suggest that DMBA-induced apoptosis of stroma cell supported pre B lymphocytes is AhR-dependent. First approach was similar to one used before for testing a possible role for the AhR and/or AhR-modulated P-450 activities in PAH-induced NF-κB activation (Hardin, Hinoshita, and Sherr, 1992; Kawabata and White, 1987; Blank *et al.*, 1987; Ladics, Kawabata ,and

White, 1991). It is based on using α-NF as a known competitive inhibitor of the AhR (Gasiewicz and Rucci, 1991; Merchant, Arellano, and Safe, 1990) and AhR-regulated cytochromes P-450 1A1 and 1A2 (Kawabata and White, 1987). The fact that α-NF pre-treatment prevents the apoptosis suggests a role for the AhR. Block of DMBA-induced apoptosis by α-NF is unlikely to be due to P-450 inhibition because other potent P-450 inhibitors had no similar effect (K. Mann *et al,* manuscript in preparation). Second, BMS2 stroma cells possessing high level of functionally active AhR are necessary for DMBA-induced apoptosis. AhR-negative pre B cells supported by rIL-7 containing media alone did not respond apoptotically to DMBA treatment. Third, AhR-deficient cell lines used as a stroma layer supporting pre B lymphocyte do not support BU-11 cells apoptosis otherwise consequent to PAH exposure (Near *et al.*, submitted).

The results of our study represent PAH toxicity to developing pre B lymphocytes and do not necessarily represent events characteristic of more mature cell lineage. The differential expression of the AhR during lymphocyte development is demonstrated by the fact that mature peripheral lymphocytes are AhR-positive (Hayashi *et al.*, 1995; Lorenzen and Okey, 1991), and their AhR is activated by TCDD (Wood *et at.*, 1993). Human tonsillar B cells (Lorenzen and Okey, 1991), human B lymphoblastoid cell lines (Waither *et al.,* 1991), and Epstein-Barr virus immortalized B cells (Masten and Shiveick, 1995) possess the AhR. More than, the same lymphocyte lineage may express different levels of the AhR depending on the activation status. For example, mature T lymphocytes activated via T cell receptor increase their AhR level (Lawrence, Leid, and Kerkvliet, 1996). This difference in AhR expression between different lymphocyte subsets may account for the difference between our conclusion and conclusions of some other authors stating that AhR ligands effect lymphocytes directly (Dooley and Holsapple, 1988; Wood *et at.*, 1993). Our work supports the previously published results that AhR ligands use the receptor to induce immunotoxicity (Holsapple *et al.*, 1991; Kerkvliet *et al.*, 1990; Morris *et al.*, 1994; White, Lysy, and Holsapple, 1985). We conclude that pre B cells do not sense PAH directly, instead they respond to the PAH-induced signal from stroma cell, a primary PAH target.

Disruption of B lymphopoiesis as an event secondary to PAH-induced changes in bone marrow stromal cells was suggested by previously published studies (King, Landreth, and Wierda, 1989). Furthermore, the required involvement of bone marrow stroma cells is not restricted just to B lineage but is common for thymic cultures as well (Kremer, Gleichmann, and Esser, 1994; Greenlee *et al.*, 1985). Treatment with TCDD of the cultures modeling developing thymocytes resulted in the loss of immature thymocytes mediated by changes in thymic stroma.

There are two imaginable scenarios how changes in stromal cells may lead to apoptosis of B cells they support. First, stromal cells could be influenced in such a way that they loose their ability to support lymphocytes. This is unlikely for DMBA-induced pre B cell apoptosis because these lymphocytes apoptose even if they are treated in the presence of rIL-7. rIL-7 is a cytokine able to support pre B cells, and DMBA does not interfere with it. If DMBA-treated stroma cells just stop delivering supporting factors to the B cells, the lymphocytes should not apoptose when supported by rIL-7. Therefore, it is more likely that stroma cells do not loose, but gain a function which is to release a

"death" signal to pre B cells. It should be the next step of our research to define what this "death" factor is. It may turn out that the factor is one of the cytokines controlled by NF-κB, or expressed by AhR ligands like TGFβ.

An important difference between the present study and the majority of research addressing AhR involvement in immunotoxicity is that other laboratories have typically used TCDD as AhR ligand. TCDD is a halogenated hydrocarbon with AhR affinity greatly exceeding that of DMBA or B[α]P (Cuthill, Wilhelmsson, and Poellinger, 1991). In our system TCDD did not induce BMS2 supported BU-11 apoptosis. Moreover, TCDD exposure interfered with DMBA-induced apoptosis. The author believes that natural or artificial ligands binding the AhR with different strength may induce varied changes of the receptor protein conformation. The variant forms of the ligated receptor could recruit distinct arrays of accessory proteins performing functions which are not similar (may be even opposite). An observation that TCDD exposure does not induce apoptosis in developing B lymphocytes but leads to apoptosis of immature T cells suggests that AhR machinery signals differently in various lymphoid cells.

For some cell lines, DMBA-induced apoptosis of B lymphocytes is not AhR-mediated. A cell line of murine B cell lymphoma, A20.1, grown without any accessory cell lines and treated *in vitro* with 10^{-5} M DMBA underwent apoptosis (Burchiel *et al.*, 1993). We could not detect AhR protein or mRNA in A20.1, so PAH induced apoptosis can not be mediated by the AhR. Remarkably, the DMBA concentration required to induce apoptosis in A20.1 is three orders of magnitude higher than needed for apoptosis induction in BMS2-supported pre B cells. It could be that there are two mechanisms of PAH apoptosis: AhR mediated, and AhR-independent. PAH induced AhR-independent apoptosis of T cell hybridomas will be discussed below.

The evolutionary conserved (Hahn and Karchner, 1995) AhR could not be designed by nature to sense DMBA and/or other fairly new compounds, but it could be evolved to control lymphocyte growth, development and/or function (Fernandez-Salguero, 1995). We believe that exogenous AhR ligands, such as PAH, allow us to gain insights the physiological role of AhR.

Fluoranthene Induces Aromatic Hydrocarbon Receptor Independent Apoptosis of Murine T Cell Hybridomas.

In this part of the present research, we tested if non-halogenated PAH influence T cells similar to how they are toxic to B lymphocytes. It was previously shown (indirectly) that the halogenated AhR ligand, TCDD, skews formation of the T cell repertoire (Silverstone *et al.*, 1994). Stimulated by previous PAH research, we tested if fluoranthene, a non-halogenated PAH, induces T cell apoptosis, and examined the potential role of AhR and Ca^{2+} influx as mediators (Karras and Holsapple, 1994; Davila *et al.*, 1995).

Fluoranthene was chosen as a model nonhalogenated PAH in our study. Cellular and molecular effects of fluoranthene are not well-studied despite it's environmental ubiquity, and ability to potentiate PAH-induced cell transformation (Rice *et al.*, 1984). Fluoranthene-induced suppression of B lymphopoesis was demonstrated in a pre B cell

model (somewhat analogous to our model described above) for as low a dose of fluoranthene as 0.05 mM (Hinoshita *et al.*, 1992). It was suspected that fluoranthene does not activate the AhR (Crespi *et al,* 1985). T lymphocyte models were provided by T cell hybridomas characterized, at least to some extent, for their T cell receptor-mediated death signals (Ju *et a*1., 1995).

The same criteria were used to assess apoptosis of T hybridoma as were described earlier for evaluation of apoptosis of pre B cells. There criteria were DNA digestion into oligonucleosome-sized fragments, dull DNA-P.I. staining, and a change in cell morphology. 12 to 14 hours of treatment of T cell hybridomas with fluoranthene resulted in apoptosis as indicated by all three techniques. This implies that apoptosis may be one mechanism of fluoranthene immunotoxicity.

The IARC report of 1983 states that 26.3 pg of fluoranthene is present in mainstream smoke of an average cigarette; it's concentration in gasoline engine exhaust is 1.0-1.6 mg/liter. High doses of fluoranthene, humans are constantly exposed to, make it a high priority goal to define which model of fluoranthene's toxicity is the most adequate and/or sensitive. The doses of fluoranthene inducing mutations in mammalian cells *in vitro* (Barfknecht *et al.*, 1981) are 10-20 times higher than the 0.1 mM concentration which induced T cell apoptosis in our study. We conclude that immunotoxicity assays are more sensitive to PAH exposure than mutational ones. Further modifications of immunotoxicity tests aimed to look for effects of even lower doses of fluoranthene are needed. Our assay detects apoptosis at a given point in time. It is likely that chronic exposures result in cumulative effects inducing toxicity at lower fluoranthene doses. Such an exposure schedule effect was observed for B[α]P-induced suppression of B cell lymphopoiesis (Hardin *et al.,* 1992). 7 days of exposure to B[α]P induced apoptosis at concentration three orders of magnitude lower than the concentration effective after 12 hours of exposure.

The data described above would be of little importance if it was a clonal artifact. To exclude this option we tested three additional hybridomas, K31H28, 12.13, and 5D5 for their ability to apoptose after treatment with fluoranthene. 5D5 was the only hybridoma tested which did not apoptose in response to fluoranthene. It is not clear what makes 5D5 fluoranthene-resistant. One can speculate that it is an absence of the same signals which make MRL-lpr/lpr-derived 5D5 hybridoma resistant to apoptosis via Fas-mediated cell death. Unlike MEI, K3lH28, or 12.13 hybridomas, 5D5 cells do not possess Fas on their surface (Ju *et al.,* 1995). It would be of interest to see which elements of a signal transduction pathways Fas- and fluoranthene-induced apoptosis have in common. Overall, these data suggests that some T cell subsets are competent to undergo fluoranthene-induced apoptosis and other lack some component(s) mediating this effect.

PAH immunotoxicity and carcinogenicity have been under scrutiny for many years and some researchers view them as "flip and flop sides of the same coin" (Davila *et al.,* 1995; Holsapple *et al.,* 1991). This notion is supported by a demonstration of AhR involvement in PAH toxicity known so far (Kerkvliet *et al.,* 1990a,b; Kawabata and White, 1987; Hardin *et al.,* 1992; Morris *t al.,* 1994). Immunotoxicity of high affinity AhR ligands were especially emphasized (Wojdani *et al.,* 1984). Our results support research arguing for existence of AhR-independent immunotoxicity (by PAH) as well

(Kerkvliet *et al.,* 1990a,b). The T cell hybridomas we have used are AhR protein and mRNA negative. We can not recall any previously published study in which PAH induced apoptosis in a cell system lacking the AhR. Our observation that unlike supporting stroma cells, pre B lymphocytes and T cell hybridomas are AhR negative, is consistent with previously demonstrated expression of AhR protein and mRNA in thymic stromal cells, but not in thymocytes (Abbott *et al.*, 1994). Consistent with the absence of the AhR in T cell hybridomas is α-naphthoflavone's inability to block fluoranthene-induced apoptosis. Additional proof that apoptosis due to fluoranthene exposure is not mediated by AhR is provided by lack of fluoranthene induced AhR nuclear translocation in Hepa-1 cells. Fluoranthene also fails to induce cytochrome P450 1A1 activity (Crespi *et al.,* 1985). It is possible that the 4S aromatic hydrocarbon binding protein, a putative alternative receptor, mediates the apoptosis (Sterling *et al.,* 1994). One of the major conclusions from these results is that PAH risk assessment- currently based on necessary AhR involvement- should be extended to reflect AhR-independent PAH toxicity.

In lymphocytes, two AhR ligands, TCDD and DMBA, induce Ca^{2+} signaling (Davila *et al.,* 1995; Holsapple *et al.,* 1991). In the present report we conclude that intracellular and/or extracellular Ca^{2+} plays a role in fluoranthene-induced apoptosis as well. This was shown by suppression of the apoptosis by chelation of extracellular Ca^{2+} ions. One can not use flow cytometry to demonstrate Ca^{2+} influx directly, because of emission spectra overlap for Ca^{2+}-binding fluorochromes (e.g. indo-1 and fluo-3) with fluoranthene and/or it's metabolites. Ca^{2+} signaling is one more similarity between fluoranthene-mediated apoptosis and apoptosis induced by lymphocyte receptor cross-linking, and other stimuli (Nicotera *et al.,* 1989). This raises the question which particular physiological signal pathways fluoranthene mimics. The present work can be continued by searching for mediators connecting fluoranthene exposure to Ca^{2+} signaling in an AhR-independent manner.

Toxicological and mechanistic approaches complement each other to form a "Möbius strip". In our research, for example, the mechanistic/biochemical observation that NF-κB is modulated by certain PAHs could mean that NF-κB-controlled functions (e.g. cardiovascular control, male reproductive activity etc.) may be vulnerable to PAH exposure. Further epidemiological and toxicological studies are needed to test this. These types of toxicity could not be under question as long as the AhR was thought to be the only PAH sensor. Another example of toxicological/pharmacological prediction coming out of our mechanistic research is that the agents arresting cells in G_0 stage of a cell cycle will reduce cell sensitivity to some PAH by decreasing the intracellular level of the AhR.

Our research demonstrates an opposite situation as well when a biochemical messenger is predicted based on toxicological data. We think that PAH-induced AhR-mediated apoptosis of the AhR-negative pre B cell growing on top of the AhR-positive stroma cells depends on a messenger which the stroma signals to a lymphocyte cell. This messenger could be a previously discovered molecule performing a new function. It could also be a new molecule. Further research should be undertaken to identify and characterized this molecule.

Finally, the dominating paradigms of toxic effect mechanisms can not be reliably extrapolated to models similar but not identical to the models they were inferred from. For example, it was contrary to our original prediction that fluoranthene-induced T cell apoptosis is Ah receptor-independent. Another class of environmental toxins which have AhR-dependent and independent mechanisms of action (depending on isoform) is PCBs. From the biochemical point of view, this raises the question of the actual mechanism of the process. From the toxicological standpoint, it is important to know what other compounds share this yet unknown mechanism.

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Abbrevations:

AhR:	Aryl hydrocarbon receptor
ARNT:	AhR nuclear translocator
α-NF:	α-naphthoflavone
B[α]P:	benzo[α]pyrene
B[*e*]P:	benzo[*e*]pyrene
bFGF:	basic Fibroblast Growth Factor
DMBA:	7,12-dimethylbenz[a]anthracene
EMSA:	electrophoretic gel mobility shift assay
EROD:	ethoxyresorufin-o-deethylase activity assay
FACS:	fluorescent activated cell sorter
HAH:	halogenated aromatic hydrocarbon
Igκ:	immunoglobulin-κ
NF-κB:	nuclear factor-κB
PAH:	polycyclic aromatic hydrocarbon(s)
PCB:	polychlorinated biphenyls
PDGF:	platelet derived growth factor
P.I.:	propidium iodide
RT-PCR:	reverse transcription polymerize chain reaction
TCDD:	2,3,7,8 tetrachlorodibenzo-*p*-dioxin
XRE:	xenobiotic-specific DNA response element

List of Cell Lines:

ME1	T hybridomas
5D5	T hybridomas
K31H28	T hybridomas
12.13	T hybridomas
Hepa-1	Hepatoma
Hepa-1 C2	Hepatoma (AhR deficient)
Hepa-1 C37	Hepatoma (AhR deficient)
BP^{r}Cl	Hepatoma (AhR deficient)
BMS2	Stroma Cells
A20.1	B lymphocytes
BU-11	B lymphocytes
3T3	Fibroblasts

ORIGINAL COMMUNICATIONS.

Article 1

Fluoranthene-Induced Apoptosis in Murine T Cell Hybridomas Is Independent of the Aromatic Hydrocarbon Receptor.

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Fluoranthene-Induced Apoptosis in Murine T Cell Hybridomas Is Independent of the Aromatic Hydrocarbon Receptor[1]

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Fluoranthene-Induced Apoptosis in Murine T Cell Hybridomas Is Independent of the Aromatic Hydrocarbon Receptor. YAMAGUCHI, K., NEAR, R., SHNEIDER, A., CUI, H., JU, S-T., AND SHERR, D. H. (1996). *Toxicol. Appl. Pharmacol.* 139, 144–152.

Recent studies suggest that environmental chemicals such as polycyclic aromatic hydrocarbons (PAH) compromise the immune system in part through the induction of programmed cell death (apoptosis). Nevertheless, mechanisms through which PAH induce apoptosis remain elusive. In particular, the role of the 8S AhR remains controversial and the nature of intracellular signal transduction in PAH-induced apoptosis remains largely undefined. To extend previous studies to the T cell compartment and to develop a clonal system in which intracellular signals leading to PAH-induced apoptosis can be dissected, the ability of fluoranthene, a ubiquitous, but less well-studied PAH, to induce apoptosis in murine T cell hybridomas was evaluated. Particular emphasis was placed on the role of the 8S AhR. The data indicate that (1) three of four hybridomas studied undergo apoptosis within 8 hr of fluoranthene exposure; (2) fluoranthene induces growth arrest concurrent with apoptosis; (3) at doses sufficient to induce lymphocyte apoptosis, fluoranthene does not induce AhR nuclear translocation in cells expressing high AhR levels; (4) fluoranthene-responsive hybridomas do not express AhR mRNA or protein; (5) the Ca^{2+} chelating agent EGTA partially inhibits fluoranthene-induced apoptosis. These results (1) indicate the immunosuppressive potential of fluoranthene; (2) support a role for apoptosis in PAH immunotoxicity; (3) demonstrate that fluoranthene-mediated T cell death and growth arrest are AhR independent; and (4) illustrate similarities between PAH- and antigen-specific receptor-mediated apoptosis. These findings encourage consideration of AhR-independent events in PAH risk assessment. © 1996 Academic Press, Inc.

Apoptosis is a physiologic death process (Kerr *et al.*, 1972) that plays a critical role in the development of the T cell repertoire in the thymic microenvironment (Ju *et al.*, 1995; Cohen, 1991; Gillette-Ferguson and Sidman, 1994) and in T and B cell peripheral tolerance (Newell *et al.*, 1990; Ucker *et al.*, 1992; Kamesaki *et al.*, 1994). Factors which modulate programmed cell death have the potential to disrupt lymphocyte repertoire development and/or to compromise immune responsiveness. Recently a number of studies have demonstrated the ability of environmental chemicals such as PAH[2] to compromise the immune system (Tomar and Kerkvliet, 1991; reviewed in Holsapple *et al.*, 1991; Davila *et al.*, 1995). At least part of this immunotoxicity may be mediated by the induction of programmed cell death in lymphocytes. While the ability of halogenated hydrocarbons to induce thymocyte apoptosis is controversial (McConkey *et al.*, 1988; Silverstone *et al.*, 1994), the ability of nonhalogenated PAH to induce apoptosis in T and B cells *in vitro* and *in vivo* is gaining support (Burchiel *et al.*, 1992, 1993; Hardin *et al.*, 1992; Hinoshita *et al.*, 1992).

In the present work the possibility that a highly bioactive but less well-studied nonhalogenated PAH, fluoranthene, is capable of inducing apoptosis in T cells was studied. Concerns for the potential immunosuppressive effects of fluoranthene arise from fluoranthene's environmental ubiquity (IARC, 1983), by its ability to suppresses B lymphopoiesis (Hinoshita *et al.*, 1992), and by its structural similarity to other immunotoxic PAH. Furthermore, the ability of this mutagenic PAH (Vaca *et al.*, 1992; LaVoie *et al.*, 1994; Wang and Busby, 1993) to potentiate the carcinogenicity of immunotoxic PAH such as B[α]P (Rice *et al.*, 1984) suggests that it may also potentiate the immunotoxicity of other PAH. Synergy with other PAH resulting in either cell transformation or immunosuppression is of added significance given the frequent association of fluoranthene with several PAH in complex mixtures (IARC, 1983).

To confirm the immunosuppressive potential of fluoranthene, to extend studies to the T cell compartment, and to develop a clonal system in which intracellular signals leading to PAH-induced apoptosis can be dissected, the ability of

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[2] Abbreviations used: ARNT, aromatic hydrocarbon nuclear translocating factor; B[α]P, benzo[α]pyrene; DMBA, 7,12-dimethylbenz[α]anthracene; PAH, polycyclic aromatic hydrocarbon(s); PI, propidium iodide; TCDD, 2,3,7,8 tetrachlorodibenzo-*p*-dioxin.

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fluoranthene to induce apoptosis in T cell hybridomas was tested. In view of the evidence that much of the bioactivity attributed to PAH, including immunotoxicity, is mediated by the 8S AhR (Hardin *et al.*, 1992; Kawabata and White, 1987; Silkworth and Vecchi, 1985; Blank *et al.*, 1987; Kerkvliet *et al.*, 1990a,b) and is Ca^{2+}-dependent, particular emphasis was placed on the role of the AhR and extracellular ions in fluoranthene-induced T cell apoptosis. Finally, since induction of apoptosis frequently affects or is affected by cell cycle (Hunter and Pines, 1994), the influence of fluoranthene exposure on cell cycle was analyzed.

MATERIALS AND METHODS

Cells. Hybridomas ME1 and K31H28 were derived by fusing sperm whale myoglobin-immunized Balb/c lymph node cells and keyhole limpet hemocyanin-immunized A/J lymph node cells respectively with the HGPRT$^-$ BW5147 fusion cell line. Hybridomas 5D5 and 12.13 were produced from separate fusions of BW5147 cells and cultured lymph node T cells from MRL-*lpr/lpr* mice (Ju *et al.*, 1995). Fusions were selected in HT containing medium and were cloned by limiting dilution. Hybridomas were routinely maintained in DMEM supplemented with 10% FCS, 100 u/ml penicillin and streptomycin, 1 mM L-glutamine (Gibco-BRL, Inc., Grand Island, NY), and 5×10^{-5} M 2-mercaptoethanol (Sigma). All hybridomas expressed high levels of the T cell receptor as assessed by flow cytometry.

Cloned bone marrow stromal cells which support pre-B cell growth were generously provided by Dr. P. Kincade (Oklahoma Medical Research Foundation) and have been previously described (Pietrangeli *et al.*, 1988). A cloned pre-B cell line was produced by culturing CD45$^+$ (B220$^+$), sIgM$^-$ pre-B cells established under Whitlock/Witte conditions (Whitlock *et al.*, 1984) on BMS2 cloned bone marrow stromal cells (Yamaguchi *et al.*, in preparation).

Hepa-1 (clone Hepa-1c1c7) cells were generously provided by Dr. J. Whitlock (Stanford University).

Treatment of cells with fluoranthene. Hybridoma T cells were maintained in log growth phase by splitting cultures 1:3 every day. Cells (10^5) were added to 5-cm^2 culture wells in 1 ml medium. Vehicle (acetone) or fluoranthene dissolved in acetone was added (final vehicle concentration = 0.1%) and cultures were harvested at various points thereafter for apoptosis or cell cycle assays. Vehicle had no effect on cultures.

Fluorescence assay for apoptosis and cell cycle. Quantitation of apoptotic cells and analysis of cell cycle were performed as previously described (Hinoshita *et al.*, 1992; Hardin *et al.*, 1992). Briefly, cells were washed in cold PBS, pelleted, and resuspended in 1.5 ml hypotonic fluorochrome solution containing 5.0 μg/ml propidium iodide (PI; Sigma Chemical Co., St. Louis, MO), 1% sodium citrate, and 0.1% Triton X-11 (Sigma). Treated cells were analyzed on a Becton–Dickinson FACScan flow cytometer. 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. The percentage of cells in G_2/S was defined as the percentage of viable cells (i.e., nonapoptotic cells) in which PI fluorescence was stronger than the G_0/G_1 peak. For analysis of cell morphology, cells were resuspended in PBS containing 10% FCS.

DNA gels. Cells (10^6) were washed and resuspended in cold Tris/EDTA buffer containing 0.2% Triton X-100. Debris and chromosomal DNA were pelleted by microcentrifugation and the supernatant was transferred to a fresh tube. After addition of 35 μl 3 M sodium acetate, fragmented DNA was extracted by phenol–chloroform extraction, precipitated with ethanol, pelleted, rinsed with cold ethanol, dried, and resuspended in TE buffer (Sambrook *et al.*, 1989). For gel electrophoresis, samples were added to loading buffer consisting of 40% sucrose in TE buffer, 1% SDS (Sigma), bromophenol blue, and 2.5 μg/ml RNase (Gibco/BRL) and loaded into dry wells of a 3.5% NuSieve agarose (FMC Bioproducts, Rockland, ME) gel in Tris acetate buffer (Sambrook *et al.*, 1989). Gels were run at 50 V for 2 hr and stained with ethidium bromide.

AhR immunoblotting. Hybridoma T or Hepa-1 cells lifted from plates by a 3-min treatment with 0.25% trypsin containing 1 mM EDTA·4Na (Gibco-BRL) were washed twice in cold 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 orthovanadate, 1 mM PMSF), and centrifuged for 15 min at 15,000*g*. Protein concentrations in supernatants were measured with a bicinchoninic acid protein assay reagent kit (Pierce, 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 hr. Proteins were transferred from gels to nitrocellulose filters (Bio-Rad, Hercules, CA) at 150 V for 1 hr or at 30 V overnight. The 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 base, pH 7.5, 0.5 M NaCl, 0.03% Tween 20). Filters were blocked with TBST buffer containing 5% dry milk. Filters were then washed twice for 5 min in TBST and incubated with monoclonal anti-AhR antibody Rpt 1 (Singh and Perdew, 1993) 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 1:6000 dilution of goat anti-mouse antibody conjugated with horseradish peroxidase (Sigma). Filters were washed twice and developed by chemiluminescence (Du Pont NEN, Boston, MA). Typical exposure times ranged from 5 to 180 sec. To approximate the sensitivity of AhR immunoblotting, it was assumed that 1 μg of Hepa-1 cytosolic protein contains 10 pg of AhR (Poland *et al.*, 1990).

Nuclei isolation. Seventy percent confluent Hepa-1 cells were treated for 3 min with 0.25% trypsin, 1 mM EDTA·4Na (Gibco), pelleted, washed twice in ice-cold PBS buffer, and resuspended in 1 ml $P_{10}EG$ buffer (10% glycerol, 0.2% Triton X-100, 8.4 M $KH_2PO_4 \cdot 3H_2O$, 10 mM EDTA, pH 7.4). Cell suspensions were rigorously pipetted. Nuclei were centrifuged for 12 min at 6000*g*. The quality of nuclei preparations was monitored by phase-contrast microscopy. Nuclei were washed twice with $P_{10}EGD$, resuspended in lysing buffer, and protein prepared for immunoblotting as described above.

RT-PCR for AhR mRNA. Whole cellular RNA was extracted from 5×10^6 cells using "RNAzol" exactly as described by the manufacturer (Leedo Medical Laboratories, Houston, TX). The integrity of RNA samples was determined by gel electrophoresis prior to RT-PCR. RT-PCR to detect AhR mRNA in 5-μg samples was performed as described by the manufacturer (Gibco/BRL) using the following 5' and 3' primers: CTGGCAATGAATTTCCAAGGGAGG and CTTTCTCCAGTCTTAATCATGCG. These primers represent sequences which bound the putative ligand-binding domain of the murine AhR (Dolwick *et al.*, 1993; Ema *et al.*, 1992). The $MgCl_2$ concentration was adjusted to 2.5 mM to maximize specific signal. Amplified DNA was electrophoresed through 3.5% NuSieve gels and DNA visualized by ethidium bromide staining. This assay detects approximately 100–200 RNA copies/reaction.

RESULTS

Fluoranthene induces apoptosis in T hybridoma cells. To determine the potential for fluoranthene to compromise the T lymphocyte compartment, ME1 T hybridoma cells were treated with vehicle or with 0.2 mM fluoranthene and the proportion of cells subsequently undergoing apoptosis was quantitated by DNA staining with propidium iodide and flow cytometric analyses of cell morphology (Hardin *et al.*,

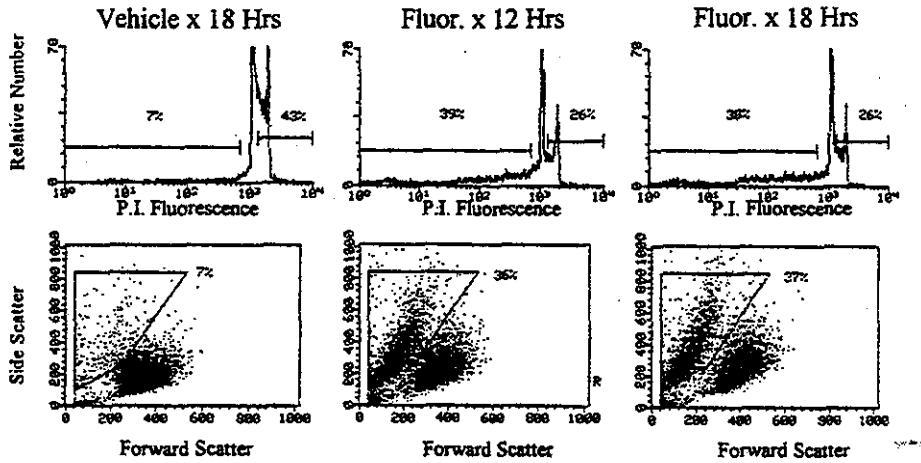

FIG. 1. Fluoranthene induces changes in DNA structure and cell morphology which are characteristic of apoptosis. Vehicle (0.1%) or fluoranthene (0.2 mM) was added to ME1 hybridoma cells in duplicate wells. Wells were pooled 12 or 18 hr later and separated into two aliquots. One aliquot was treated with 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 (the left most regions in each histogram) and are relatively small and granular (enclosed region of each dot plot). The regions on the right of each histogram represent cells in the S/G_2 phases of the cell cycle. The numbers over these regions indicate the percentage of cells in the region relative to the total number of viable, nonapoptotic cells.

1992). Data from one representative experiment (from over 30 total) are presented in Fig. 1. Relatively few (7%) vehicle-treated cells exhibited a dull PI staining pattern characteristic of cells undergoing apoptosis. Similarly, few control cells (7%) 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 by both DNA staining and morphologic parameters, increased to 36–39% within 12 hr of fluoranthene exposure. No further increase in the percentage of apoptotic cells was noted at 18 hr (Fig. 1) or at later time points (up to 48 hr; data not shown). Titration experiments demonstrated significantly elevated levels of apoptotic cells 24–48 hr after addition of as little as 0.1 mM fluoranthene and a trend toward increased apoptosis at half that dose (Fig. 2). These data suggest that fluoranthene exposure rapidly induces T cell apoptosis.

To confirm that the changes in PI staining and cell morphology correlated with DNA fragmentation characteristics of apoptosis, ME1 cells were treated with vehicle or with fluoranthene for 4, 8, 12, or 18 hr and their DNA extracted and electrophoresed in 3.5% agarose gels. An aliquot of cells was treated with dexamethasone for 12 hr as a positive control. Consistent with results shown in Fig. 1, low but detectable levels of DNA digestion into oligonucleosome-sized fragments were observed in vehicle-treated cells (Fig. 3A, lane 1). While no further digestion was seen 4 hr after fluoranthene treatment, DNA digestion increased dramatically when cells were treated with fluoranthene for 8 hr (lane 3). No further increase in DNA fragmentation was detectable after 12 hr (Fig. 3B, lanes 2–4).

Apoptosis induced with 0.2 mM peaked at about 40% and did not increase after 8–12 hr, suggesting that a subset of cells is refractory to this fluoranthene dose. Since induction of apoptosis by a number of stimuli is cell cycle dependent (Hunter and Pines, 1994), it appeared possible that the failure to see an apoptosis increase after 8–12 hr reflected the ability of fluoranthene to induce growth arrest in addition to apoptosis and that growth-arrested cells are resistant to fluoranthene-mediated apoptosis signals. If this hypothesis is correct, it would be predicted that the percentage of cells in the G_2/S phases of the cell cycle will decrease in the presence of fluoranthene and that release from growth arrest, e.g., by removal of fluoranthene, will regenerate dividing cells which undergo programmed cell death in response to a second

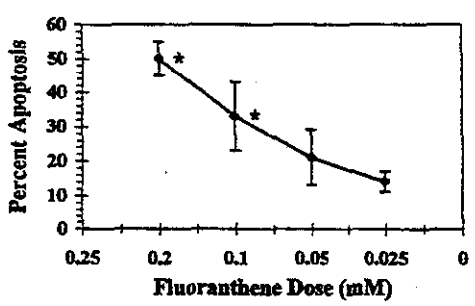

FIG. 2. Dose response of ME1 hybridoma cells to fluoranthene. Vehicle (0.1%) or fluoranthene (0.025–0.2 mM) was added to ME1 hybridoma cells in duplicate wells. Wells were pooled 24–48 hr later and the percentage of cells undergoing apoptosis was quantitated by PI staining. Data are pooled from 14 independent experiments. An asterisk indicates a significant increase in apoptosis relative to vehicle controls, $p < 0.05$.

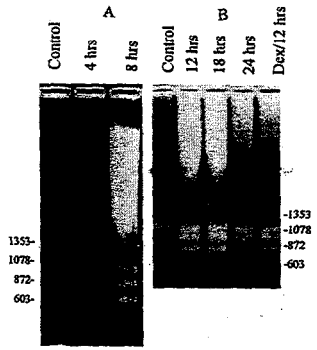

FIG. 3. Fluoranthene rapidly induces DNA fragmentation in ME1 hybridoma T cells. Vehicle (0.1%), fluoranthene (0.2 mM), or dexamethasone (0.01 mM) was added to ME1 hybridoma cells in duplicate wells. Cells were harvested 4, 8, 12, and 18 hr later. DNA was extracted and electrophoresed in 3.5% agarose gels. Data presented in B were obtained from cells also used to generate the data in Fig. 1. Sizes of DNA fragments in base pairs are indicated in the margins. Data from a representative experiment are presented.

exposure to fluoranthene. Indeed, fluoranthene consistently reduced the percentage of viable cells in G_2/S from 52 ± 5 to 29 ± 3% (Fig. 1, Table 1; "Pretreatment: Nothing"). Cells treated with fluoranthene for 16 hr, washed, and cultured without fluoranthene for 30 hr (and with vehicle for an additional 16 hr) recovered from growth arrest and apoptosis as indicated by an increase in the percentage of G_2/S cells (Table 1: 29 ± 3% vs 59 ± 1%) and a decrease in the percentage of apoptotic cells (49 ± 11% vs 16 ± 4%). Treatment of these cells with fluoranthene again resulted in a significant decrease in G_2/S cells (from 59 ± 1 to 40 ± 2%) and a parallel increase in apoptosis (from 16 ± 4 to 60 ± 7%). Culture of these cells ["Pretreatment: Fluoranthene (twice)"] for 78 hr without fluoranthene (and with vehicle for an additional 16 hr) again resulted in the recovery of G_2/S cells (40 ± 2 to 55 ± 1%) and the concomitant emergence of fluoranthene-responsive cells (17 ± 13% vs 45 ± 15% apoptosis). The percentage of dead cells, as assessed by trypan blue exclusion, paralleled (but was generally lower than) the percentage of apoptotic cells in each group and the total number of cells (viable + nonviable) recovered was essentially the same in vehicle- and fluoranthene-treated groups. While removal of all residual fluoranthene in these experiments is unlikely, depletion by washing cells and changing the media was sufficient to rescue cells from both the growth arrest and apoptosis induced by exogenous fluoranthene. These results are consistent with the hypothesis that fluoranthene induces both apoptosis and growth arrest and that recovery from fluoranthene-mediated growth arrest results in the emergence of apoptosis-sensitive cells.

Fluoranthene-induced apoptosis in additional T cell hybridomas. To generalize results, three additional T cell hybridomas were tested for their ability to undergo apoptosis in response to fluoranthene. Hybridomas ME1, K31H28, and 12.13 exhibited similar sensitivity to fluoranthene (Table 2). In contrast, hybridoma 5D5 was significantly less responsive than the other three hybridomas. Growth arrest was observed in ME1, K31H28, and 12.13 but not in 5D5 cells (data not shown). These results indicate that, while common to other T cell hybridomas, fluoranthene-induced apoptosis and growth

TABLE 1
Cells Recovering from Fluoranthene-Induced Growth Arrest Regain Fluoranthene Sensitivity[a]

	Nothing		Fluoranthene (once)		Fluoranthene (twice)	
Pretreatment	Vehicle	Fluoranthene	Vehicle	Fluoranthene	Vehicle	Fluoranthene
% G_2/S	52 ± 5	29 ± 3**	59 ± 1	40 ± 2**	55 ± 1	42 ± 3**
% apoptosis	15 ± 5	49 ± 11*	16 ± 4	60 ± 7*	17 ± 13	45 ± 15*

[a] ME1 hybridoma cells (10^5) were treated with vehicle or 0.2 mM fluoranthene for 16 hr. An aliquot of cells (Pretreatment: nothing) was harvested and stained with PI in hypotonic buffer. An aliquot of 10^5 fluoranthene-treated cells [pretreatment: fluoranthene (once)] was washed, cultured 30 hr without fluoranthene, reseeded, and treated with vehicle or fluoranthene for 16 hr. Aliquots were harvested and stained with trypan blue or PI in hypotonic buffer. An aliquot of 10^5 fluoranthene-treated cells (pretreatment: fluoranthene (twice)] was washed, cultured without fluoranthene for 78 hr, reseeded, and treated with vehicle or fluoranthene for 16 hr. Cells were harvested and stained with trypan blue or with PI in hypotonic buffer. The total numbers of cells recovered after 16 hr in vehicle or fluoranthene-treated groups (approximately 4×10^5) did not differ significantly. Data (arithmetic means ± standard errors) were averaged from three experiments. The percentage apoptosis was calculated as the percentage of the total population with sub-G_0/G_1 staining. The percentage of cells in the G_2/S phases of cell growth was calculated as the percentage of viable cells (i.e., nonapoptotic cells) expressing levels of DNA higher than that in the G_0/G_1 peak.

* Significant level of apoptosis, $p < 0.03$.

** Significant decrease in the percentage of cells in G_2/S, $p < 0.03$.

	ME1	K31H28	12.13	5D5
Vehicle (% apoptosis)	8 ± 1	8 ± 3	7 ± 1	6 ± 2
Fluoranthene (% apoptosis)	37 ± 4*	37 ± 4*	34 ± 5*	14 ± 1***

" T cell hybridomas ME1, K31H28, 12.13, and 5D5 were cultured in duplicate wells in the presence of 0.1% vehicle or 0.2 mM fluoranthene for 24 h. Wells were pooled and the percentage of cells undergoing apoptosis quantitated by the PI/flow cytometry method. Data (arithmetic means ± standard errors) were averaged from 7 to 10 experiments.

* Significant level of apoptosis relative to vehicle controls, $p < 0.001$.

** Significant difference relative to apoptosis observed in fluoranthene-exposed ME1, K31H28, or 12.13 cells, $p < 0.006$.

arrest may require certain characteristics not exhibited by all T cell hybridomas.

Fluoranthene-induced T cell apoptosis is not Ah receptor-dependent. It has been suggested that aromatic hydrocarbons mediate biologic activities, including immunosuppression, through the AhR (White *et al.*, 1985; Morris *et al.*, 1994; Hardin *et al.*, 1992; Silkworth and Vecchi, 1985). However, AhR-dependent toxicity in lymphoid cells remains controversial, particularly in view of the paucity of direct proof that the AhR is present and functional in lymphocyte subsets and in light of the possibility that immunosuppression may occur secondary to toxicity in accessory cells which support lymphocyte growth and/or function (Kremer *et al.*, 1994; Greenlee *et al.*, 1985). Furthermore, the ability of fluoranthene in particular to bind to and activate the AhR has not been adequately addressed. Three approaches were undertaken to assess the potential role of the AhR in fluoranthene-induced T hybridoma cell apoptosis: (1) evaluation of fluoranthene-induced AhR nuclear translocation, (2) analysis of AhR protein levels in T hybridoma cells, and (3) analysis of AhR mRNA levels in T hybridoma cells.

Fluoranthene's ability to induce nuclear translocation in cells which express high AhR levels, i.e., Hepa-1 liver cells (Singh and Perdew, 1993), was studied by immunoblotting nuclear protein. Consistent with their relative binding affinities for the AhR (Bigelow and Nebert, 1982) and their ability to induce P450IA1 activity (Crespi *et al.*, 1985), B[α]P and, to a lesser extent, DMBA induced nuclear AhR in Hepa-1 cells (Fig. 4). However, fluoranthene consistently failed to induce detectable levels of nuclear AhR at doses sufficient to induce apoptosis in T hybridoma cells (0.2 mM), suggesting that, within the limits of detectability, fluoranthene cannot significantly induce AhR nuclear translocation, even in cells expressing high AhR and ARNT levels. These results do not formally exclude the possibility that fluoranthene induces AhR-mediated second messenger signaling (Enan and Matsumura, 1995) without nuclear translocation.

FIG. 4. Fluoranthene does not induce AhR nuclear translocation. Hepa-1 cells were treated with DMBA (4×10^{-4} M), fluoranthene (2×10^{-4} M), or B[α]P (2×10^{-4} M) for 1.5 hr. Nuclear protein was extracted and 160 μg immunoblotted with monoclonal anti-AhR antibody (Rpt-1). The predominant 95- to 110-kDa AhR band is shown. Data from a representative experiment are presented.

The fact that the AhR cannot play a role in T hybridom: apoptosis was further supported by the absence of detectable AhR protein in ME1, K31H28, or 12.13 cells by Western immunoblotting (Fig. 5). It should be noted that studies with titered concentrations of Hepa-1 cell-derived AhR indicate that AhR immunoblotting is sensitive to approximately 500 pg of AhR. Furthermore, using the more sensitive technique of RT-PCR, AhR mRNA was readily detectable in Hepa-1 but not ME1 (Fig. 6), K31H28, or 12.13 cells (not shown). Interestingly, high levels of AhR mRNA were detected i bone marrow stromal cells which support pre-B cell growth, while little or no AhR mRNA was detected in a pre-B cell line. Given results with the highly sensitive RT-PCR assay (100–200 mRNA copies), the absence of detectable AhR

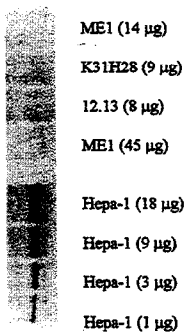

FIG. 5. T hybridomas ME1, K31H28, and 12.13 do not express detectable AhR protein. Total cellular protein was extracted from Hepa-1 cells and from ME1, K31H28, and 12.13 T hybridoma cells and immunoblotted with monoclonal anti-AhR antibody (Rpt-1). Titered concentrations of Hepa-1 protein were used to assess the relative sensitivity of the immunoblotting assay (approximately 500 pg AhR protein). For this calculatic it was assumed that 1 μg of Hepa-1 cytosolic protein contains 10 pg c AhR (Poland *et al.*, 1990). The predominant 95- to 110-kDa AhR band is shown. Data from a representative experiment are presented.

FIG. 6. ME1 T cells do not express detectable levels of AhR mRNA. Total cellular RNA was extracted from Hepa-1 and ME1 cells, from a bone marrow stromal cell line (BMS2), and from a long-term pre-B cell line. The RNA was reverse transcribed and subjected to PCR to amplify DNA coding for the putative AhR ligand binding domain as described under Materials and Methods. Amplified DNA was electrophoresed in 1.5% agarose gels.

protein in T hybridoma cells by immunoblotting, and the failure of fluoranthene to activate detectable levels of AhR in Hepa-1 cells, it is concluded that fluoranthene cannot and does not activate the AhR in the course of inducing apoptosis in T hybridoma cells.

Fluoranthene-induced apoptosis is partially ion-dependent. It has been shown that Ca^{2+} plays an important signal transduction role in lymphocyte apoptosis (Nicotera *et al.*, 1989). In particular, an influx of extracellular Ca^{2+} (Burchiel *et al.*, 1992) and the release of Ca^{2+} from intracellular stores (Holsapple, 1995) appear to play important roles in aromatic hydrocarbon-mediated lymphocyte death and/or dysfunction (Krieger *et al.*, 1994; Davila *et al.*, 1995; Karras and Holsapple, 1994). To determine if extracellular ion flux participates in AhR-independent signal transduction leading to PAH-induced apoptosis, ME1 cells were cultured in the presence of the ion chelator EGTA, fluoranthene, or EGTA + fluoranthene for 16–24 hr. Cells were then harvested and assayed for apoptosis. EGTA alone (1–2 mM) had no effect on the low level of background apoptosis observed in ME1 cultures (Table 3). While fluoranthene induced significant levels of apoptosis, 1 or 2 mM EGTA significantly inhibited apoptosis. These results demonstrate that the presence of extracellular ions is required for maximal apoptosis and is consistent with a role for Ca^{2+} in signal transduction leading to PAH-induced apoptosis.

DISCUSSION

In recent years it has become clear that PAH are capable of suppressing the immune system at multiple levels, including suppression of T cell, B cell, and macrophage function and induction of lymphocyte death (reviewed in Davila *et al.*, 1995). Studies with two of the best characterized PAH, B[α]P and 7,12-DMBA, indicate that at least some of the immunotoxicity attributed to these PAH is mediated by the induction of B and T cell apoptosis (Burchiel *et al.*, 1992, 1993; Hardin *et al.*, 1992; Hinoshita *et al.*, 1992). PAH induction of lymphocyte apoptosis suggests an overall diminution of immune competence. Furthermore, since programmed cell death, manifest as apoptosis, is a critical component in the development and maintenance of both T and B lymphocyte repertoires, the ability of PAH to activate cell suicide suggests that PAH may interfere with the normal development and maintenance of both T and B lymphocyte lineages. This possibility is supported by the recent studies of Silverstone *et al.* in which TCDD exposure, although not directly shown to induce apoptosis, was shown to alter the developing T cell repertoire (Silverstone *et al.*, 1994). A common mechanistic thread in many of the studies on PAH immunotoxicity is the potential role of the AhR and/or Ca^{2+} in signaling lymphocyte dysfunction or death (Karras and Holsapple, 1994; Davila *et al.*, 1995).

In the present studies, the possibility that a less well-characterized but equally important PAH, fluoranthene, could similarly induce apoptosis was assessed. These studies were motivated by fluoranthene's environmental ubiquity, its ability to potentiate PAH-induced cell transformation (Rice *et al.*, 1984), its likely inability to activate the AhR (Crespi *et al.*, 1985), and its ability to suppress B lymphopoiesis (Hinoshita *et al.*, 1992). A panel of T cell hybridomas was chosen to assess fluoranthene's immunotoxic potential since cloned target cells are amendable to studies on the biochemistry of PAH-mediated signal transduction and because we have delineated at least some of the T cell receptor-mediated death signals in these hybridomas (Ju *et al.*, 1995).

Three criteria were applied to define the induction of apoptosis: (1) a change in cell morphology, (2) a decrease in DNA–propidium iodide staining, and (3) the digestion of DNA into oligonucleosome-sized (i.e., multiples of 180–200 bp) fragments. By all three criteria, fluoranthene induced

TABLE 3
EGTA Inhibits Fluoranthene-Induced Apoptosis[a]

Vehicle	2 mM EGTA	Percentage apoptosis fluoranthene	Fluoranthene + 1 mM EGTA	Fluoranthene + 2 mM EGTA
5 ± 1	5 ± 1	46 ± 8	30 ± 7* (39%)	22 ± 2** (59%)

[a] ME1 cells were cultured in the presence of 0.1% vehicle, EGTA, 0.2 mM fluoranthene, or fluoranthene + EGTA for 16–24 hr. Duplicate wells were pooled and the percentage of cells undergoing apoptosis was quantitated by the PI/flow cytometry method. Data (arithmetic means ± standard errors) were averaged from 14 independent experiments. Numbers in parentheses indicate the percentage suppression of fluoranthene-induced apoptosis.

Significant reduction in apoptosis relative to the fluoranthene only group, $*p < 0.01$ or $**p < 0.001$, respectively.

apoptosis in T cell hybridomas within a relatively short time, 8–12 hr, thereby implicating apoptosis as a contributing mechanism of fluoranthene immunotoxicity.

Titration studies demonstrate that 0.1 mM fluoranthene rapidly induces T cell apoptosis. The ability of a slightly lower fluoranthene dose (0.05 mM) to induce pre-B cell apoptosis (Hinoshita *et al.*, 1992) suggests that, while both T and B cell compartments respond to fluoranthene, developing B cells may be more sensitive than mature lymphocytes. Nevertheless, the doses of fluoranthene required to induce T cell apoptosis are still 10–20 times lower than those required to induce mutations in mammalian cells *in vitro* (Barfknecht *et al.*, 1981). Thus, immunotoxicity assays appear to be more sensitive indicators of the bioactivity of this relatively common PAH (e.g., 26.3 μg/cigarette in mainstream smoke, 1.0–1.6 mg/liter gasoline in engine exhaust; IARC, 1983) than mutational assays. It should be noted that these assays measure apoptosis at a given point in time. Therefore, the cumulative effects of chronic fluoranthene exposure over longer periods of time at the doses employed are likely to be underestimated when extrapolating from the current studies. In support of this conclusion is the observation that the dose of B[α]P capable of suppressing B cell lymphopoiesis after a 7-day culture period is three logs lower than that required to visualize the likely mechanism of toxicity, apoptosis, after 12 hr (Hardin *et al.*, 1992).

Apoptosis was accompanied by a change in the distribution of cells within the cell cycle. That is, a preferential decrease in the percentage of viable cells in the G_2/S phases of the cell cycle and a corresponding increase in G_0/G_1 cells were observed during the first 8–12 hr of fluoranthene exposure. These results suggest that fluoranthene-mediated apoptosis is preferentially activated at the G_1 to S checkpoint. The fact that fluoranthene induced at least partial growth arrest in addition to apoptosis and that dividing cells were particularly sensitive to apoptotic signals was supported by the finding that treatment of cells with fluoranthene yielded a population of fluoranthene-resistant cells, a majority of which exhibited characteristics of quiescent cells (i.e., G_0/G_1 levels of DNA). Removal of cells from fluoranthene resulted in an increase in G_2/S (i.e., dividing) cells and a parallel regeneration of cells capable of undergoing apoptosis in response to fluoranthene. Induction of apoptosis concomitant with growth arrest is reminiscent of findings in other systems. For example, apoptosis induced in B lymphocytes with anti-B cell receptor antibody (Yao and Scott, 1993) and in the same panel of T cell hybridomas employed herein with anti-CD3 antibody or through Fas ligand (Ju *et al.*, 1995; data not shown) is accompanied by growth arrest. Collectively, the results suggest that fluoranthene induces intracellular death signals through mechanisms analogous to those induced through lymphocyte receptor cross-linking and involving regulation of the cell cycle. Indeed, the similarity in the outcome of fluoranthene exposure and T cell receptor cross-linking in these hybridomas, i.e., the induction of apoptosis, parallels the studies of Archuleta *et al.* in which intracellular signaling following DMBA exposure mimicked some aspects of T cell receptor signaling (Archuleta *et al.*, 1993). Furthermore, the results presented here emphasize that multiple mechanisms of T cell immunotoxicity, e.g., induction of apoptosis and cell growth, may be effected by fluoranthene.

Three of the four hybridomas tested readily induced the cell death program and were growth arrested in response to fluoranthene. The fact that the fourth hybridoma, 5D5, was relatively resistant suggests that fluoranthene-induced apoptosis requires specific intracellular events that may be compromised in resistant cells. We have demonstrated that the MRL-*lpr/lpr*-derived 5D5 hybridoma, but not ME1, K31H28, or 12.13 hybridomas, is also resistant to Fas-mediated cell death. This resistance correlates with the presence of functional Fas on ME1, K31H28, or 12.13 but not on 5D5 cells (Ju *et al.*, 1995; data not shown). While these observations suggest a possible role for Fas in fluoranthene-induced T cell death, defects in other parts of the complex signaling pathway leading to cell death are possible. In any case, results with all of the hybridomas indicate that fluoranthene activates at least part of a common signaling pathway leading to programmed cell death and that resistant cell lines, such as 5D5, may prove useful in defining this pathway. The possible role of Fas in fluoranthene-induced T cell death is under study.

The observation that the immunotoxicity of a given PAH generally correlates with its carcinogenicity has led to the hypothesis that mechanisms of immunotoxicity and carcinogenicity overlap (Davila *et al.*, 1995; Holsapple *et al.*, 1991). The most direct support of this hypothesis is the finding that the AhR influences immunotoxicity and transformation in a number of systems (Kerkvliet *et al.*, 1990a,b; Kawabata and White, 1987; Hardin *et al.*, 1992; Morris *et al.*, 1994), particularly when the immunotoxicant has a high affinity for the AhR (Wojdani *et al.*, 1984). However, an obligatory role for the AhR in all forms of immunotoxicity appears unlikely (Kerkvliet *et al.*, 1990a,b). In the present studies it was determined that none of the T cell hybridomas tested expressed detectable levels of AhR protein or mRNA. To our knowledge, this is the first demonstration of PAH-induced lymphocyte apoptosis in a clearly AhR^- cell line. Interestingly, a pre-B cell line, used as a control for these experiments, expressed little or no AhR mRNA (Fig. 6) and no detectable AhR protein (data not shown). In contrast, high levels of AhR were detected in bone marrow stromal cells. Although AhR has been detected in human tonsillar B cells (Lorenzen and Okey, 1991), human B lymphoblastoid cell lines (Waither *et al.*, 1991), and Epstein–Barr virus immortalized B cells (Masten and Shiverick, 1995), our results are more

consistent with those of Abbott *et al.* who demonstrated the expression of AhR mRNA and protein primarily in thymic stromal cells, not in thymocytes (Abbott *et al.*, 1994). The absence of detectable AhR mRNA or protein in nonactivated T cell hybridomas and the failure of α-naphthoflavone, an AhR antagonist and cytochrome P450 inhibitor, to block fluoranthene-induced apoptosis (data not shown) demonstrate that fluoranthene immunotoxicity need not be mediated by the AhR. Furthermore, fluoranthene's failure to induce detectable AhR nuclear translocation (Fig. 4) and cytochrome P450IA1 activity (Crespi *et al.*, 1985) suggests that fluoranthene cannot mediate AhR-dependent immunotoxicity. These observations suggest that the paradigm used to assess the relative risk of human exposure to PAH should be modeled to include the possibility that some PAH may exert their effects in an AhR-independent fashion. The results do not exclude the possibility that the biologic activity of fluoranthene or other PAH is mediated by a putative alternative receptor, the 4S aromatic hydrocarbon binding protein (Sterling *et al.*, 1994).

While the AhR appeared not to contribute to intracellular death signals in the present system, extracellular ions, probably Ca^{2+}, did have a role. Previous studies with DMBA and TCDD, low- and high-affinity AhR ligands, respectively, have implicated a role for intracellular and/or extracellular Ca^{2+} in PAH signaling in lymphocytes (Davila *et al.*, 1995; Holsapple *et al.*, 1991). In the present report we demonstrate that chelation of extracellular ions suppresses fluoranthene-induced apoptosis. [Direct demonstration of the influx of Ca^{2+} by flow cytometry was precluded by overlap in the emission spectra of Ca^{2+}-binding fluorochromes, including indo-1 and fluo-3, with the emission spectra of fluoranthene and/or its metabolites (data not shown)]. Thus, Ca^{2+} is likely to be a common element in aromatic hydrocarbon signaling regardless of the involvement of the AhR. The implicated role for Ca^{2+} in fluoranthene-mediated apoptosis is consistent with a role for extracellular Ca^{2+} in apoptosis induced by a number of death signals (Nicotera *et al.*, 1989) and again suggests that fluoranthene-mediated death signals are similar to those induced through physiologic stimuli such as lymphocyte receptor cross-linking. The identification of AhR^- cloned T hybridoma cells which activate the cell death program in response to fluoranthene will facilitate investigations into AhR-independent PAH signaling.

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Article 2

Expression of the Aryl Hydrocarbon Receptor Is Regulated by Serum and Mitogenic Growth Factors in Murine 3T3 Fibroblasts.

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Expression of the Aryl Hydrocarbon Receptor Is Regulated by Serum and Mitogenic Growth Factors in Murine 3T3 Fibroblasts*

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The aryl-hydrocarbon receptor (AhR) is a ligand-activated transcription factor that mediates biological responses to planar aromatic hydrocarbons such as benzo[α]pyrene. However, no endogenous physiological ligand for the AhR has been identified. Since the AhR regulates bioactivity to common environmental pollutants, and since it is predicted to play an important physiological function, we have investigated the expression of the AhR during the cell cycle of murine 3T3 fibroblasts. We show here that stimulation of growth-arrested 3T3 cells with serum results in increased expression of AhR protein. Serum-induced expression of AhR in synchronized, serum-stimulated cells occurs at the onset of DNA synthesis (S phase) and is maximal at time points corresponding to late S phase. Transient transfections with an AhR-promoter-luciferase construct demonstrate that reporter gene transcription from the AhR promoter is regulated in a serum-dependent manner. Serum-dependent induction of AhR expression is prevented by an inhibitor of tyrosine kinase activity. Ligand-activated growth factor receptors (platelet-derived growth factor receptor basic fibroblast growth factor receptor) as well as an ectopically expressed tyrosine kinase (the v-Src oncoprotein) induce AhR expression in the absence of serum. Therefore, tyrosine kinase signaling is both necessary and sufficient for induction of AhR expression. Studies with the G_1 blocker sodium butyrate show that the signal transduction pathways mediating serum-stimulated progression through the cell cycle are distinct from those that induce AhR expression. These data suggest that transcriptional regulation of the AhR is important in determining cellular sensitivity to the actions of AhR ligand(s) and that the AhR may play a role during the cellular proliferative response.

The aryl hydrocarbon receptor (AhR)[1] is a ligand-activated transcription factor of the basic helix-loop-helix family that displays high affinity binding to certain planar aromatic compounds. Such compounds include polycyclic aromatic hydrocarbons (typified by benzo[α]pyrene, or B[α]P) and halogenated aromatic hydrocarbons (typified by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin). Polycyclic aromatic hydrocarbons such as B[α]P are generated during the combustion of fossil fuels and are present in tobacco smoke and smoked meats. Halogenated aromatic hydrocarbons such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin are formed as contaminants during the manufacture of several commercial products, including the chlorophenols. These chemicals are persistent high level environmental pollutants and cause a variety of toxic and carcinogenic effects that are largely mediated by activation of the AhR (for reviews see Refs. 1 and 2 and references therein).

The unliganded AhR exists in the cytosol, in a complex with the 90-kDa heat shock protein (HSP 90). Upon binding to aryl hydrocarbons, the AhR dissociates from HSP 90 and translocates to the nucleus, where it is thought to form a complex with the aryl hydrocarbon receptor nuclear transporter (ARNT) protein (3, 4). The ligand-activated AhR·ARNT complex binds to specific enhancer sequences (termed xenobiotic-response elements; XREs), present within the promoter region of aromatic hydrocarbon-inducible genes. The most widely studied AhR-responsive genes are the AhR-inducible members of the P450 cytochrome family. Ligand activation of the AhR results in transcriptional activation of genes of the P4501 subfamily, specifically P4501A1/1A2 and P4501B1 (1, 2, 5). The DNA-bound AhR·ARNT heterodimer is thought to facilitate the recruitment of other transcription factors to the promoter, thereby promoting transcription (1, 2). However, the ligand-activated AhR can also inhibit transcription of certain genes by binding to XREs that overlap other positive regulatory elements (6).

The AhR is widely expressed and is postulated to play a role in normal growth and development based upon patterns of AhR expression in developing mouse embryos (7, 8). Although it is likely that a physiological ligand for the AhR does exist, no such molecule has yet been identified. Nevertheless, inappropriate activation of the AhR by aromatic hydrocarbons induces a variety of cell-specific effects. These include increased proliferation (*e.g.* parenchymal cells, epithelial cells of the urinary tract, the intestine, and the interfollicular epidermis), inhibition of differentiation (*e.g.* the generative cells of the gastric glands) as well as tumor promotion in experimental animals (Refs. 1 and 2 and references therein). Thus, AhR activation can have profound effects on cell growth and differentiation. These data support the notion that the AhR plays a part in normal growth and development and that deregulation of AhR signal transduction pathways by aromatic hydrocarbons interferes with normal growth processes.

Regulation of expression of the AhR is a potentially important mechanism for determining the physiological responsiveness to AhR ligands during normal growth and development.

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[1] The abbreviations used are: AhR, aryl hydrocarbon receptor; B[α]P, benzo[α]pyrene; ARNT, aryl hydrocarbon receptor nuclear transporter; XRE, xenobiotic-response elements; PDGF, platelet-derived growth factor; bFGF, basic fibroblast growth factor; EMSA, electrophoretic mobility shift assay.

Moreover, regulated expression of the AhR may underlie the cell-specific responsiveness to AhR ligands and may be an important determinant of aryl hydrocarbon-induced toxicity. Here we report that expression of the AhR is stringently regulated during the course of the mammalian cell cycle. These data establish a further link between the AhR and cell proliferation and lend support to other studies implying a role for the AhR during the life cycle of mammalian cells.

Murine 3T3 fibroblasts have provided a valuable cell system for the study of growth factor-regulated signal transduction and cell cycle-regulated events (for reviews see Refs. 9 and 10). 3T3 fibroblasts are immortal, nontransformed cell lines, originally derived from mouse embryos. The growth properties and growth factor requirements of 3T3 cells have been well characterized. Moreover, much is known regarding the molecular mechanisms that regulate cell cycle progression in these cells. For exponential growth, 3T3 fibroblasts require exogenously added growth factors, usually supplied by supplementation of the growth medium with 10% donor calf serum. Upon serum withdrawal, 3T3 cells undergo growth arrest and remain in a quiescent state termed G_0. However, when stimulated with fresh serum or certain defined mitogenic growth factors (such as platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF)), the cells synchronously re-enter the G_1 phase of the cell cycle. G_1 is defined as the time interval between G_0 (or mitosis) and the initiation of DNA synthesis (S phase) and is characterized by a cascade of growth factor-stimulated signaling events including growth factor-induced synthesis of second messenger molecules, activation of small GTP-binding proteins, protein kinase cascades, and the transcriptional activation of "immediate early" and "delayed response" genes whose protein products are thought to be necessary for progression through the cell cycle (reviewed in Refs. 9–12). Thus, the cell cycle comprises a complex program of temporally ordered events that mediate cellular responses to external mitogenic stimuli.

We have investigated the expression of the AhR during the 3T3 fibroblast cell cycle. We show here that expression of the AhR is regulated at the transcriptional level in response to serum and purified growth factors during the course of the cell cycle. These data suggest that regulation of AhR expression may be an important mechanism for sensitizing cells to AhR ligands and strengthen the idea that the AhR serves an important function during normal cell growth and development.

MATERIALS AND METHODS

Cells and Culture—Swiss 3T3 cells were obtained from the ATCC and were grown in Dulbecco's modified Eagle's medium supplemented with penicillin and streptomycin containing 10% donor calf serum. To induce growth arrest, cells were placed in culture medium containing 0.5% serum for 24 h. v-src-expressing Swiss 3T3 cells were obtained as described previously (13).

Fluorescence-activated Cell Sorter Analysis and [^{3}H]Thymidine Incorporation Assays—These experiments were carried out as described previously (13).

Preparation of Cytosolic Extracts and Nuclei for Immunoblotting—Monolayers of Swiss 3T3 cells in 10-cm culture dishes were rinsed with 10 ml of phosphate-buffered saline. The washed monolayers were detached by the addition of 1.5 ml of 10 mM Tris (pH 7.0), 150 mM NaCl, 1 mM EDTA (TNE) for 10 min at room temperature. Detached cells were scraped off the dish, placed in a microcentrifuge tube, and centrifuged at 10,000 × *g* for 10 s. The washed cell pellets were lysed in 100 μl of lysis buffer (20 mM Hepes (pH 7.4), 150 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.25% Nonidet P-40), vortexed vigorously, and incubated on ice for 5 min. The lysates were centrifuged at 10,000 × *g* for 5 min. The resulting supernatants (cytosolic extracts) were removed and frozen at −70 °C prior to immunoblotting. Nuclear pellets were resuspended and washed in 200 μl of lysis buffer and then repelleted at 10,000 × *g*. The washed nuclear pellets were resuspended in 100 μl of lysis buffer containing 2 units of RQ DNase I (Promega). Chromatin was digested on ice for 10 min. Digested nuclei were then frozen at −70 °C prior to SDS-polyacrylamide gel electrophoresis.

Anti-AhR Antisera—All experiments shown were performed with two independently generated antibodies. Dr. Gary Perdew kindly provided us with a monoclonal anti-AhR antibody (clone RPT-1). A polyclonal antibody to recombinant murine AhR was also purchased from Biomol. Both antisera recognized a single 95-kDa band on immunoblots that was blocked by excess recombinant AhR. Identical results were obtained using both antibodies.

SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting—25-μg aliquots of each sample were denatured by heating to 90 °C in SDS-reducing buffer and were separated by electrophoresis on 7.5% SDS-polyacrylamide gels. After transfer to nitrocellulose, the filters were probed with antisera to murine AhR. The blots were developed using an ECL kit (Amersham Corp.).

Transient Transfections—Exponentially growing cultures of 3T3 cells were transfected with 10 μg of the appropriate plasmid DNA plus 20 μg of salmon sperm carrier DNA by calcium phosphate co-precipitation. After 12 h, the transfection medium was removed and replaced with fresh culture medium. 12 h later, the cells were placed in culture medium containing 10 or 0.5% serum. 18 h later, the transfected monolayers were washed with phosphate-buffered saline and detached with TNE as described above. Cell extracts were prepared by detergent lysis and were assayed for luciferase activity using a commercially available kit (Promega).

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assays (EMSAs)—To prepare nuclear extracts, monolayers of 3T3 cells were first rinsed and then scraped into 5 ml of phosphate-buffered saline and pelleted by centrifugation at 1000 × *g* for 5 min. Washed cells (approximately 10^7) were resuspended in 5 ml of hypotonic ice-cold lysis buffer (20 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamidine, 0.5 mM dithiothreitol), left on ice for 10 min, and then homogenized with 10 up/down strokes of a hand-held glass/glass homogenizer. The lysate was centrifuged at 5000 × *g* for 5 min. After aspirating the supernatant, the nuclear pellet was resuspended in approximately 0.3 ml of salt extraction buffer (20 mM Hepes (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamidine, 0.5 mM dithiothreitol). Extraction of nuclear proteins was carried out by incubation on ice for 20 min. The salt-extracted nuclei were removed by centrifugation at 10,000 × *g* for 10 min. The resulting supernatants containing the salt-extracted nuclear proteins were stored at −70 °C prior to EMSA analysis. XRE binding reactions were performed in a volume of 30 μl containing 10 mM Hepes (pH 7.9), 50 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 5% glycerol, 5 μg of poly(dI:dC), and 5 μg of nuclear extract protein. All components were incubated on ice for 10 min prior to addition of double-stranded ^{32}P-labeled oligonucleotide probe specifying the XRE (14). The incubation was continued for a further 20 min after the addition of the probe. Reaction mixes were loaded onto a 6% polyacrylamide gel and electrophoresed at 200 V. Protein-DNA complexes with retarded electrophoretic mobility were detected by autoradiography of the dried gel.

RESULTS

Growth Arrest Is Associated with Elevated AhR Expression—Exponentially growing cultures of Swiss 3T3 cells were placed in fresh growth medium containing 10% serum or were transferred to medium containing reduced (0.5%) serum for 24 h. Fluorescence-activated cell sorter analysis of propidium iodide-stained nuclei confirmed that the cells underwent growth arrest following serum starvation, as evident by reduced levels of cells in S and G_2 + M relative to cells in 10% serum (Fig. 1*A*). Immunoblot analysis of detergent lysates from parallel cultures of fibroblasts with two independent anti-AhR antibodies (see "Materials and Methods") indicated greatly reduced expression of a 95-kDa immunoreactive protein in cytosol from serum-starved cells relative to cells growing in 10% serum (Fig. 1*B*). Although prolonged development of immunoblots did eventually enable detection of the 95-kDa band in extracts from serum-starved cells (see, for example, Figs. 4, 6, 7, and 8), serum stimulation consistently resulted in 4–10-fold induction of levels of this protein.

To establish that the immunoreactive 95-kDa protein corresponded specifically to the AhR (and not a related PAS protein)

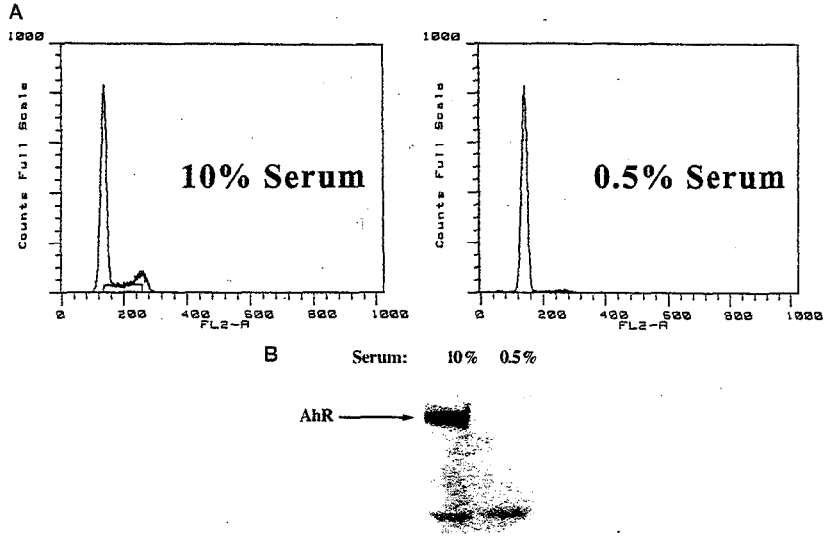

FIG. 1. **Growth arrest is associated with reduced expression of the AhR.** Parallel cultures of fibroblasts were placed in fresh medium containing 10 or 0.5% serum for 24 h. Cells were then examined for growth state by fluorescence-activated cell sorter analysis of propidium iodide-stained nuclei (*A*) or for expression of AhR by immunoblotting (*B*), as described under "Materials and Methods." The percentages of cells in G_0/G_1, S, and G_2 + M phases of the cell cycle were 75.0%, 14.7%, and 10.3%, respectively, in exponentially cycling cells, and 96.8%, 1.4%, and 1.7%, respectively, in serum-starved fibroblasts. Data are representative of at least five separate experiments.

we tested the effects of a specific AhR-activating ligand on the subcellular localization and cellular levels of the protein. Exponentially growing 3T3 cells were treated with the AhR ligand B[α]P. Nuclear and cytosolic extracts were prepared at frequent intervals following B[α]P treatment. Cell extracts were then separated by SDS-polyacrylamide gel electrophoresis and probed with anti-AhR antisera (Fig. 2). As would be expected for the AhR, brief (0.5-h) exposure to 1 μM B[α]P resulted in nuclear translocation of the immunoreactive 95-kDa species, and prolonged (4-h) treatment with ligand resulted in complete down-regulation of this protein (Fig. 2). These data are similar to the findings of Swanson and Perdew, who also demonstrated ligand-induced down-regulation of the AhR (15), and further confirm the identity of the 95-kDa immunoreactive protein in our experiments as the AhR.

We tested whether the growth state-associated changes in AhR expression were of sufficient magnitude to affect the extent of ligand-dependent AhR nuclear translocation. Exponentially growing 3T3 cells (in 10% serum) and serum-starved cells were stimulated for 45 min with B[α]P. Proteins present in nuclear extracts from the cells were tested for XRE binding activity by EMSA. As shown in Fig. 3, nuclear extracts from proliferating cells contained increased amounts of XRE binding activity following B[α]P stimulation relative to growth-arrested fibroblasts. These experiments demonstrate that the state of growth arrest resulting from serum starvation is associated with reduced expression of the AhR and consequent reduced cellular responsiveness to an AhR ligand.

We examined the kinetics of AhR induction following readdition of 10% serum to quiescent (serum-starved) cultures of cells. Immunoblot analysis of AhR expression at various time

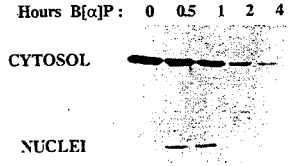

FIG. 2. **Effect of an AhR ligand on subcellular distribution and cellular levels of AhR.** Exponentially growing cultures of fibroblasts were treated with 1 μM benzo[α]pyrene (added as a 1000 × stock in Me_2SO) for 0, 0.5, 1, 2, and 4 h. At each time point following treatment, cytosolic and nuclear extracts were prepared and analyzed for AhR content by immunoblotting as described under "Materials and Methods." The *upper panel* shows cytosolic AhR levels, and the *lower panel* shows the AhR content of nuclei from an equivalent number of cells. This experiment was repeated three times with similar results.

intervals following the addition of serum showed that serum-induced expression of AhR was detectable after a lag period of approximately 13 h and was maximal by 18 h (Fig. 4*A*). By comparison, the onset of DNA synthesis (determined by measurements of [^{3}H]thymidine incorporation into genomic DNA) began approximately 13 h following serum stimulation of quiescent cells and was complete after 22–24 h (Fig. 4*B*). Therefore, AhR expression was induced at the G_1/S phase boundary and peaked in late S phase.

Mechanism of AhR Induction by Serum—Serum-dependent induction of the AhR protein may have potentially resulted from increases in steady state levels of the AhR mRNA. However, AhR transcripts were undetectable by RNA blot analysis

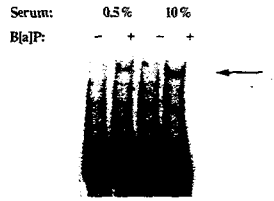

FIG. 3. **Ligand-dependent AhR nuclear translocation in growth-arrested and proliferating fibroblasts.** Parallel cultures of fibroblasts were placed in fresh medium containing 10 or 0.5% serum for 24 h. Cultures were treated with 1 μM benzo[α]pyrene (added as a 1000 × stock in Me_2SO) for 45 min. Nuclear extracts were prepared from the cells as described under "Materials and Methods." EMSA binding reactions were performed by incubating nuclear extracts with a double-stranded oligonucleotide probe specifying the XRE. The position of the B[α]P-induced band with retarded mobility is indicated by the *arrow*. This experiment was repeated twice with similar results.

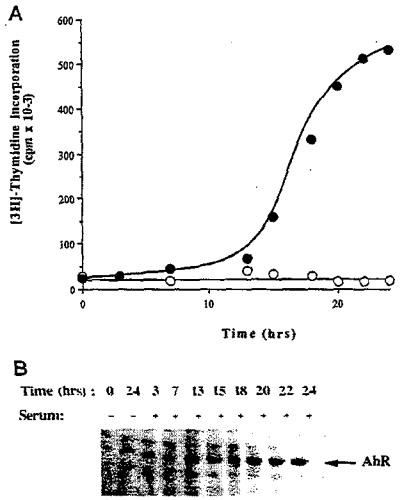

FIG. 4. **Kinetics of induction of AhR expression and DNA synthesis following serum-stimulation.** Cultures of fibroblasts were made quiescent by starvation in 0.5% serum for 24 h. The growth-arrested cultures were left untreated or were given 10% serum for the indicated times. Cells were assayed for entry into S phase by determinations of [^{3}H]thymidine incorporation (*A*) or for expression of the AhR by immunoblotting of cytosolic extracts (*B*). Each data point in *A* represents the mean of duplicate determinations that differed by less than 10%. Error bars are omitted for clarity. ○, −serum; ●, +serum. These data are representative of three separate experiments.

of samples of total RNA from Swiss 3T3 cells, and the inherent difficulties in accurate quantification of oligo(dT)-cellulose-selected transcripts precluded meaningful comparisons between different RNA samples. Therefore, we directly tested whether the increased expression of AhR protein in serum-stimulated Swiss 3T3 cells was due to serum-dependent transcription of the AhR gene using a reporter gene driven by the AhR promoter. Cultures of Swiss 3T3 cells were transiently transfected with the plasmid p5K600f, which contains 600 base pairs of the

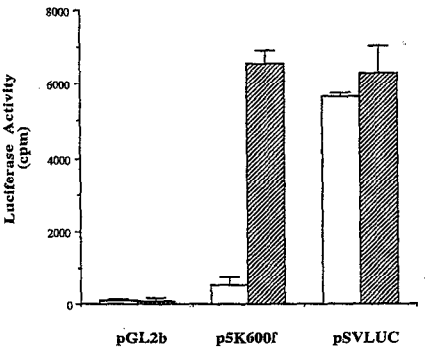

FIG. 5. **Serum-dependent AhR promoter-driven reporter gene activity.** Exponentially growing cultures of Swiss 3T3 cells were transiently transfected with the indicated plasmids by calcium phosphate co-precipitation. After 12 h, the transfected cells were fed with fresh medium containing 10% serum (▨) or were placed in culture medium containing 0.5% serum (□). 18 h later, the cells were harvested, and cytosolic extracts were assayed for luciferase activity as described under "Materials and Methods." Plasmid p5K600f contains 600 base pairs of the promoter region of the murine AhR gene upstream of a luciferase reporter gene; pGL2b is a promoterless luciferase construct; pSV2LUC contains the luciferase gene inserted downstream of a strong constitutive promoter. The values shown are means of duplicate determinations. These data are representative of two separate experiments.

promoter region of the murine AhR gene upstream of a luciferase reporter gene (16). The transfected cells were fed with fresh medium containing 10% serum or were placed in culture medium containing 0.5% serum. After 18 h, the cells were harvested, and cytosolic extracts were assayed for AhR promoter-driven luciferase activity. As shown in Fig. 5, extracts from p5K600f-transfected cells maintained in 0.5% serum expressed reduced levels of luciferase activity (greater than 90% reduction) relative to cells that were given 10% serum. By contrast, cytosolic extracts from cultures of cells that were transfected in parallel with a promoterless luciferase construct (pGL2b) or a vector containing the luciferase gene downstream of a strong constitutive promoter (pSV2LUC) did not show such serum-dependent changes in luciferase activity (Fig. 5). These data show that serum-dependent expression of the AhR results, at least in large part, from transcriptional regulation.

Tyrosine Kinase Signaling Is Necessary and Sufficient for AhR Induction—Serum comprises a poorly defined mixture of growth-stimulatory (as well as growth-inhibitory) factors. Serum-induced mitogenesis in 3T3 cells is thought to result in large part (but not entirely) from the action of polypeptide growth factors (such as PDGF) present in serum (9, 10). Consequently, serum stimulation of fibroblasts results in ligand occupancy and activation of growth factor receptors of the tyrosine kinase family (including the PDGF receptor). Downstream events resulting from ligand activation of growth factor receptors in 3T3 cells include activation of other intracellular tyrosine kinases (members of the *src* family; see Ref. 17), as well as elevated levels of signaling molecules (such as diacylglycerol, Ca^{2+}, and cAMP), which mediate activation of appropriate serine/threonine kinases (protein kinase C, calmodulin-dependent kinase, and cAMP-dependent protein kinase, respectively). These intracellular signaling events are thought to contribute to the cellular mitogenic response (reviewed in Refs. 9 and 10). Since our experiments indicated a correlation between serum-dependent progression through the cell cycle

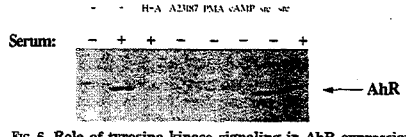

FIG. 6. **Role of tyrosine kinase signaling in AhR expression.** Quiescent cultures of Swiss 3T3 cells or v-*src*-expressing Swiss 3T3 fibroblasts (*src*) were treated with (+) 10% serum, or were left untreated (−) for 18 h. Some cultures received 0.5 μg/ml herbimycin-A (*H-A*), 2 μM calcium ionophore (A23187), 100 nM phorbol 12-myristate 13-acetate (*PMA*), or a combination of 1.75 mM dibutyryl cAMP and 0.5 mM 3-isobutyl-1-methylxanthine (*cAMP*) as indicated on the *figure*. Cytosolic extracts were prepared as described under "Materials and Methods." 100 μg of cytosolic protein from each sample was separated by SDS-polyacrylamide gel electrophoresis and analyzed for AhR expression by immunoblotting. These data are representative of three separate experiments.

and induction of AhR expression, we investigated the role of these individual signaling events in AhR induction.

Quiescent cultures of 3T3 cells were stimulated with 10% serum for 18 h in the absence or presence of the tyrosine kinase inhibitor herbimycin-A. As shown in Fig. 6, herbimycin-A inhibited serum-induced AhR expression. As expected, the concentration of herbimycin-A used in these experiments (0.5 μg/ml) prevented serum-induced DNA synthesis but did not result in cell death during the course of the experiment (not shown). Therefore, tyrosine kinase signaling was necessary for serum-dependent AhR expression. To directly test whether tyrosine kinase activity could induce AhR expression we ectopically expressed the v-Src oncoprotein in Swiss 3T3 cells. As shown in Fig. 6, the v-Src-expressing Swiss 3T3 cells expressed constitutively high levels of AhR, even under conditions of serum-starvation. By contrast, the parental Swiss 3T3 fibroblasts only expressed high levels of AhR after serum stimulation (Fig. 6).

Since protein kinase C, cAMP-dependent protein kinase, and calcium/calmodulin-dependent proteins are downstream targets of activated tyrosine kinase receptors (10), we investigated the potential role of these effectors in AhR induction. Separate cultures of quiescent fibroblasts were stimulated with 100 nM phorbol 12-myristate 13-acetate (a protein kinase C activator), 2 μM A23187 (a calcium ionophore that elevates intracellular calcium levels), or a combination of 1.75 mM dibutyryl-cAMP (a cell-penetrant cAMP analogue) and 0.5 mM 3-isobutyl-1-methylxanthine (a cyclic nucleotide phosphodiesterase inhibitor). 18 h later, cytosolic extracts were prepared and analyzed for AhR expression by immunoblotting. As shown in Fig. 6, none of the treatments induced detectable levels of AhR expression, although phorbol 12-myristate 13-acetate and dibutyryl-cAMP/3-isobutyl-1-methylxanthine both elicited a mitogenic response in parallel assays of [^{3}H]thymidine incorporation (data not shown), as has been reported by other investigators (10).

Therefore, activation of serine/threonine kinase activity (protein kinase C, cAMP-dependent protein kinase, and Ca^{2+}/calmodulin-dependent protein kinase) was insufficient to induce AhR expression. However, tyrosine kinase signaling was specifically required for induction of AhR expression in response to serum, and a constitutively active tyrosine kinase (v-Src) was sufficient to confer high level expression of AhR in the absence of serum.

Since certain defined fibroblast mitogens (PDGF or FGF) activate receptors of the tyrosine kinase family (and are known to activate c-Src, see Ref. 17), we asked whether they would also induce AhR expression. Separate cultures of serum-starved 3T3 cells were stimulated with serum, PDGF, and bFGF. The mitogen-stimulated cells were then analyzed for AhR expression (by immunoblotting) and for entry into the cell

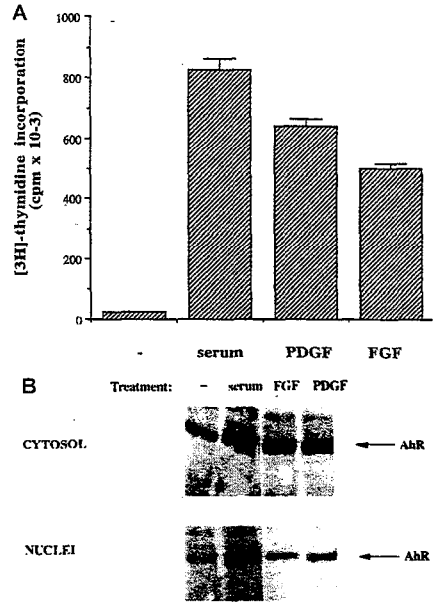

FIG. 7. **Induction of AhR expression by defined fibroblast growth factors.** Cultures of fibroblasts were made quiescent by starvation in 0.5% serum for 24 h. The growth-arrested cultures were left untreated or were given serum (10%), PDGF-BB (10 ng/ml), or bFGF (5 ng/ml). 24 h later, the fibroblasts were assayed for entry into S phase by determinations of [^{3}H]thymidine incorporation (*A*) or assayed for expression of the AhR by immunoblotting of cytosolic and nuclear fractions (*B*). Data points in *A* are means of duplicate determinations. These data are representative of three separate experiments.

cycle (by measurements of [^{3}H]thymidine incorporation). PDGF and bFGF were effective mitogens, as shown by their ability to induce [^{3}H]thymidine incorporation in serum-starved 3T3 cells (Fig. 7*A*). Both PDGF and bFGF induced AhR expression, albeit to lesser levels than were induced by serum (Fig. 7*B*). Therefore, defined fibroblast mitogens were able to induce cytosolic AhR expression. We also performed immunoblot analysis on preparations of washed nuclei from quiescent and serum/growth factor-treated cells. As shown in Fig. 7*B*, nuclei from mitogen-treated cells also contained increased levels of AhR relative to nuclei from quiescent cells. Therefore, the amount of AhR present in the nuclear compartment may be regulated by serum and mitogens during the course of the proliferative cell cycle.

Cell Cycle Progression Is Dissociable from AhR Induction—Since our experiments indicated a good correlation between the mitogenic potential of serum/growth factors and their abilities to induce AhR expression, we asked whether progression through the cell cycle was necessary for AhR induction. Therefore, we tested the effect of the cell cycle inhibitor sodium butyrate on cell cycle progression and AhR expression. Cultures of serum-starved 3T3 fibroblasts were stimulated with serum in the presence or absence of 2 mM sodium butyrate and assayed for entry into the cell cycle (by determining [^{3}H]thy-

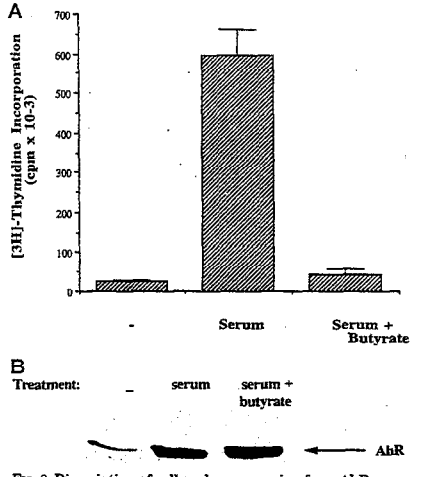

FIG. 8. **Dissociation of cell cycle progression from AhR expression.** Cultures of fibroblasts were made quiescent by starvation in 0.5% serum for 24 h. The growth-arrested cultures were left untreated or were given serum (10%) with or without 2 mM sodium butyrate. 24 h later, the fibroblasts were assayed for entry into S phase by determinations of [^{3}H]thymidine incorporation (*A*) or assayed for expression of the AhR by immunoblotting of cytosolic fractions (*B*). Data points in *A* are means of duplicate determinations. These data are representative of three separate experiments.

midine incorporation) or tested for induction of AhR expression (by immunoblot analysis). As shown in Fig. 8*A*, 2 mM sodium butyrate abolished serum-stimulated DNA synthesis, indicative of an effective G_1 block. Cell viability assays indicated no toxicity or cell death (not shown). However, serum-dependent induction of AhR expression was unaffected by the presence of sodium butyrate (Fig. 8*B*). Therefore, progression to S phase and G_2 was not necessary for serum-dependent induction of AhR expression. These data show that the serum-induced signal transduction pathways necessary for AhR expression can be dissociated from those that are required for cell cycle progression.

DISCUSSION

We have shown here that the expression of the AhR gene is transcriptionally induced by serum in murine 3T3 fibroblasts. Many of the genes induced by serum treatment of 3T3 fibroblasts are essential components of the genetic program resulting in DNA synthesis and cell division, in fibroblasts as well as in other cell types.

Serum comprises a mixture of growth-stimulatory as well as growth-inhibitory factors. As with other serum-induced genes (*e.g.* c-*myc*, c-*fos*, c-*jun*, *egr-1*), expression of the AhR is also induced by an oncogene (v-*src*) and by purified fibroblast mitogens such as PDGF and FGF (9–12). Therefore, AhR induction following serum-stimulation most likely results from mitogenic growth factors present in serum. Indeed, PDGF is known to be a major mitogenic factor in serum. However, cell cycle progression is not required to induce AhR expression, as shown by dissociation of serum-induced AhR expression and cell cycle progression with the G_1 inhibitor sodium butyrate.

Genes induced by serum growth factors in cultured fibroblasts are broadly categorized as immediate early, delayed early, or late based upon their kinetics of induction following serum/growth factor stimulation of quiescent cells (9–12). Immediate early genes are induced early in G_1 even in the presence of protein synthesis inhibitors (*e.g.* c-*myc*, c-*fos*), whereas delayed early genes are expressed later in G_1 (but prior to S phase) and require new protein synthesis (*e.g.*, ornithine decarboxylase). Late genes are those activated at or after the onset of DNA synthesis. The AhR, therefore, must be considered a late gene, since its expression peaks after the onset (and completion) of DNA synthesis.

Like the AhR, many other serum/growth factor-induced proteins are transcription factors (*e.g.* c-*myc*, c-*fos*, c-*jun*). The protein products of many immediate early genes are transcription factors that are themselves thought to mediate expression of delayed early genes and late genes, thereby achieving an ordered sequential program of transcriptional events. Interestingly, the AhR promoter contains binding sites for transcription factors that are expressed earlier in the cell cycle, namely AP-1 (composed of c-*fos* and c-*jun* dimers) and c-*myc*. It is noteworthy that, like the AhR, c-*myc* expression is elevated in exponentially growing cultures of fibroblasts relative to growth-arrested cells (18, 19) and is induced in response to serum as well as purified mitogenic growth factors such as PDGF and FGF (20). The possibility exists, therefore, that the AhR promoter is a physiologically relevant target for the c-*myc* proto-oncogene. More detailed analysis of the AhR promoter is under way to identify the *cis* acting elements and *trans*-acting factors that mediate serum-dependent transcription of the AhR gene.

Induction of AhR expression during the replicative program of 3T3 cells most likely serves to sensitize proliferating cells to the actions of AhR ligands. Indeed, our EMSA analysis demonstrates that B[α]P stimulation results in greater nuclear accumulation of the AhR in proliferating 3T3 cells relative to growth-arrested fibroblasts. Moreover, our finding that the AhR is present in nuclei of serum/growth factor-stimulated cells suggests that nuclear translocation and perhaps even transcription factor activity of the AhR is directly modified by mitogens.

Mouse embryos transcribe AhR mRNA (7, 8), and AhR −/− "knockout" mice are reported to be developmentally impaired (21), although the latter study is now controversial (22). Those studies suggest that the AhR plays an important role in normal growth and development. The 3T3 fibroblasts used in our studies were derived from Swiss mouse embryos. Our finding that expression of the AhR is transcriptionally regulated by mitogenic stimuli (serum and purified growth factors) suggests that this transcription factor may play a role in embryonic cell proliferation and differentiation. Phillips *et al.* (23) have found that the AhR ligand 2,3,7,8-tetrachlorodibenzo-*p*-dioxin can inhibit terminal adipocyte conversion of 3T3-L1 fibroblasts. Interestingly, the 3T3-L1 fibroblast subline used in the latter study was originally derived from Swiss 3T3 cells (24). Therefore, AhR activation can directly influence the balance between cell proliferation and differentiation. It is likely to be significant that this critical growth-regulatory gene is itself subject to stringent transcriptional control.

In the living organism, fibroblasts and their proliferation in response to growth factors play important roles in organogenesis, the maintenance of connective tissue, and wound healing. Our finding that the AhR is expressed in a regulated manner in these cells raises the possibility that the AhR is involved in these vital physiological processes. Moreover, many of the serum-induced genes originally identified in fibroblasts are now known to play ubiquitous roles in growth and differentiation. It

is likely, therefore, that the findings described here will be of general significance.

Acknowledgments—We thank Drs. Christopher A. Bradfield and Gary Perdew for the generous gifts of the AhR promoter-luciferase construct and anti-AhR antibody, respectively.

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Article 3

Activation of the Aryl Hydrocarbon Receptor/Transcription Factor and Bone Marrow Stromal Cell-Dependent preB Cell Apoptosis.

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) α-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.**

The 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-1β, TGF-α, and TGF-β 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

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[4] Abbreviations used in this paper: AhR, aryl hydrocarbon receptor; Arnt, aromatic hydrocarbon receptor nuclear translocator protein; B[a]P, benzo[a]pyrene; DMBA, 7,12-dimethylbenz[a]anthracene; α-NF, α-naphthoflavone; PAH, polycyclic aromatic hydrocarbon; PI, propidium iodide; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; XRE, xenobiotic-specific DNA response elements; RT-PCR, reverse transcriptase-polymerase chain reaction; TE, Tris-EDTA buffer.

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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 $KH_2PO_4 \cdot 3H_2O$, 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.

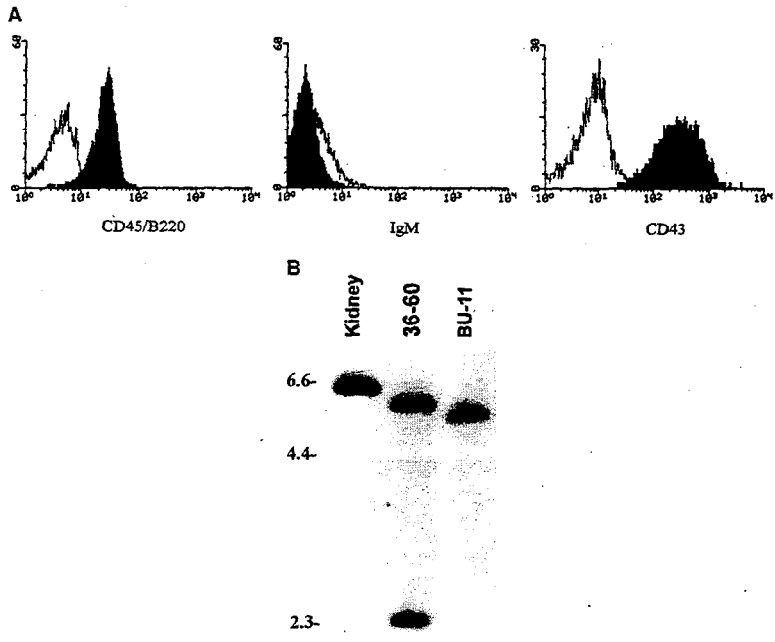
In situ hybridization

^{35}S-radiolabeled AhR riboprobes were generated using T7 (sense) and SP6 (antisense) promoters with linearized Xba and *Hind*III 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



URE 1. Characterization of the BU-11 cell line. *A*, BU-11 cells were harvested from adherent monolayers of BMS2 cells (<1% BMS2 cell tamination) and stained with fluorochrome-labeled isotype control Abs or Abs specific for CD45/B220, CD43, or surface IgM. (*B*), DNA was acted from an aliquot of BU-11 cells, murine kidney cells, or the 36-60 B cell hybridoma; electrophoresed; transferred to nylon filters; and ted with a JH-specific probe.

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

T-PCR to detect AhR mRNA in 5-μg samples was performed as de- ed by the manufacturer (SuperScript preamplification system, Life nologies) with $MgCl_2$ concentration adjusted to 2.5 mM to maximize fic signal and using the following AhR primers: CTGGCAAT TTTCCAAGGGAGG and CTTTCTCCAGTCTTAATCATGCG. ers were chosen to enclose the sequence that contains the putative ie AhR ligand-binding domain (2, 3). AhR mRNA was reverse tran- ed and aliquots were added to known titered amounts of the linearized c cDNA. Amplified DNA (10 μl of 100 μl; 35 cycles) was electro- sed through 3% gels (3:1 NuSieve:LE agarose, FMC Bioproducts) NA visualized by ethidium bromide. AhR cDNA was assumed to the amount of mimic added when the density of the AhR band ed that of the mimic (39).

hern blot analysis of heavy chain rearrangements

tures for Southern blotting and hybridization using the JH probe ave been previously described in detail (40) with the exceptions that d nylon filters were used and that the DNA transfer was done using e transfer as described by the manufacturer (GeneScreen Plus, Du- ?EN 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 paucicional 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

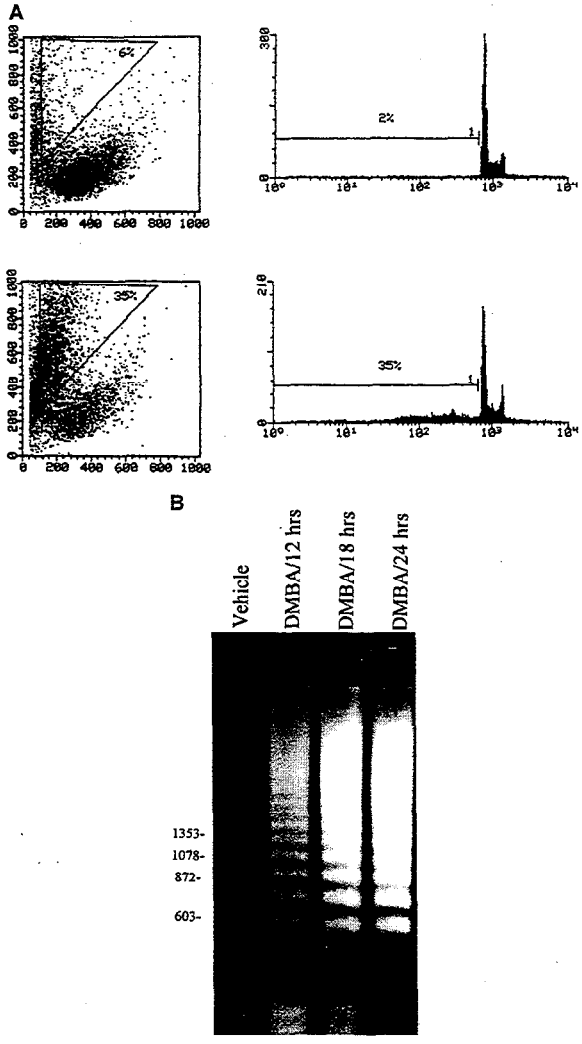


FIGURE 2. DMBA induces apoptosis in BU-11 cells. Vehicle (0.1%) or DMBA (10^{-4} M) was added to BU-11 cells in duplicate wells. *A*, Wells were pooled 18 h later and separated into two aliquots. One aliquot was treated with PI in hypotonic buffer for DNA analyses (histograms) and the other aliquot was 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. 2*B*); i.e., DNA extracted from BU-11 cells treated with 10^{-4} M DMBA

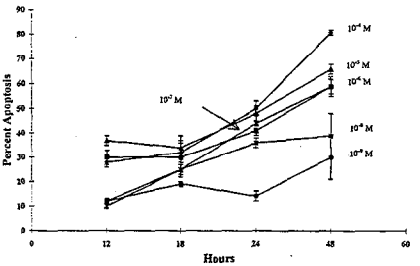


FIGURE 3. Kinetics and dose response of BU-11 cells to DMBA. Vehicle (0.1%) or DMBA (10^{-4}–10^{-9} M) was added in duplicate to cultures of BU-11 cells maintained on BMS2 stromal cells. From 12 to 48 h later BU-11 cells were harvested and stained with PI, and the percentage of cells undergoing apoptosis 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.

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

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

Inhibitor	Vehicle	DMBA 10^{-4} M	DMBA 10^{-5} M	DMBA 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.

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. 4*C*).

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. 6*A*). 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. 6*B*). 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% 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.

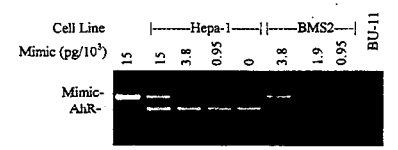


FIGURE 5. Quantitative RT-PCR for AhR mRNA in BMS2 and BU-11 cells. BU-11 cells were passed three to four times while maintained for 7 days in rIL-7 to insure the absence of contaminating BMS2 cells. RNA was extracted from Hepa-1, a liver hepatoma line (1), BMS2, or BU-11 cells, reverse transcribed and 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.

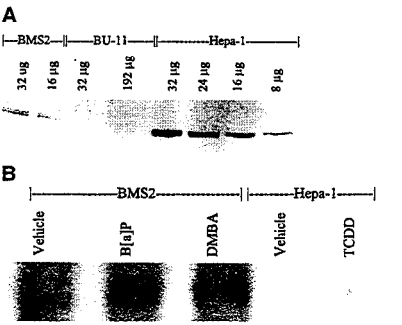


FIGURE 6. BMS2 but not BU-11 cells express functional AhR. Total cellular protein from untreated BMS2, freshly isolated BU-11 cells or Hepa-1 cells (*A*) or nuclear protein from BMS2 or Hepa-1 cells treated 60 min previously with vehicle-, 10^{-4} M B[a]P-, DMBA- or 10^{-8} M TCDD (*B*) was extracted, electrophoresed, transferred to nitrocellulose filters, and immunoblotted with monoclonal (RPT-1) anti-AhR Ab. For nuclear protein immunoblots, 15 µg of BMS2 nuclear protein and 5 µg 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,

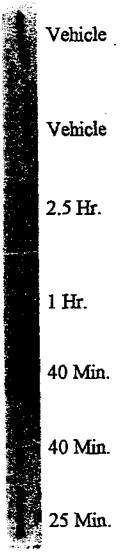


FIGURE 7. DMBA induces a loss of total AhR in BMS2 cells. BMS2 cells were treated with vehicle or 10^{-5} M DMBA for the periods indicated. Cells were harvested, washed, protein extracted, electrophoresed, transferred to nitrocellulose filters, and immunoblotted with monoclonal (RPT-1) anti-AhR Ab. The predominant 95- to 100-kDa AhR band is shown.

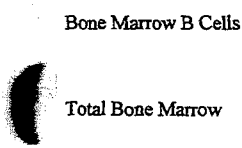


FIGURE 8. AhR is not detectable in primary bone marrow B cells. Bone marrow cells were expunged from the femurs of mice. Monocytes and stromal cells were depleted by adherence on culture dishes. Bone marrow B cells were then enriched on anti-CD45/B220 Ab-coated petri dishes. B cells were harvested, washed, 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.

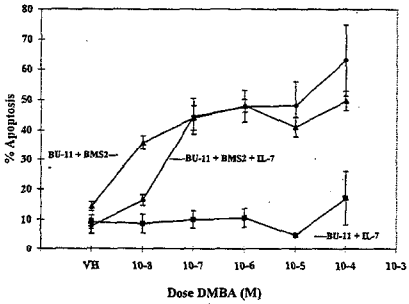


FIGURE 9. BMS2 cells are required for DMBA-induced preB cell apoptosis. Cultures of BU-11 cells maintained on BMS2 cells (BMS2 + BU-11), BU-11 cells maintained 10 to 14 days with rIL-7 in the absence of BMS2 cells (BU-11 + rIL-7), and BU-11 cells maintained on BMS2 cells with rIL-7 (BU-11 + BMS2 + rIL-7) 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, α-NF completely blocks DMBA-induced apoptosis (Table I). α-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 α-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-β 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-β, 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 $BCL\text{-}X_L$ 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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Article 4

Activation of the Aryl Hydrocarbon Receptor/Transcription Factor Induces NF-kB/Rel.

Activation of the Aryl Hydrocarbon Receptor/Transcription Factc

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Running Title: PAH-induced NF-κB/Rel

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Abstract

NF-κB is a nuclear transcription factor implicated in the regulation of immuno-receptors, cytokines, and proto-oncogenes. Most recently, modulation of NF-κB has been associated with programmed cell death. Polycyclic aromatic hydrocarbons (PAH) are a class of ubiquitous environmental toxins which both induce cell transformation and suppress immune responsiveness. Our laboratory has demonstrated that PAH induce programmed cell death in immature B lymphocytes through activation of the aryl hyrdrocarbon receptor/transcription factor (AhR). In the present study, we tested the hypothesis that PAH-mediated AhR activation induces NF-κB activity, a result which would implicate an AhR-NF-κB axis in a number of cell processes. Using a panel of murine hepatoma cells expressing varied and defined levels of AhR activity, and electrophoretic mobility shift assays to assess Igκ light chain promotor binding activity, it was demonstrated that: 1) two tested PAH, benzo[*a*]pyrene (B[*a*]P), and 7,12-dimethylbenz[a]anthracene (DMBA), induce NF-κB activity in AhR^+ hepatoma cells; 2) PAH-induced, NF-κB binding complexes contain p50 and p65 subunits; 3) a known inhibitor of AhR activity, α-naphthoflavone, prevents PAH-induced NF-κB activation; 4) benzo[*e*]pyrene, a B[*a*]P congener which weakly binds the AhR and which is proportionately less bioactive, does not activate NF-κB; 5) PAH do not activate NF-κB in AhR^- hepatoma mutants or mutants defective in AhR signaling machinery; 6) PAH induce NF-κB activity in a hepatoma mutant defective in AhR-regulated, P-4501A1 enzyme activity. These results support the hypothesis that AhR activation induces a transcriptionally active (p50/p65) NF-κB dimer and suggest a multitude of biologic responses to PAH mediated by the AhR and NF-κB.

Introduction.

Much research has focused on intracellular signaling mediated by the ubiquitous transcription factor NF-κB. Prior to activation, cytosolic NF-κB consists of at least three proteins, a dimer of two subunits of the NF-κB/Rel family (p50, p52, p65, c-Rel, Rel B) and an inhibitor of NF-κb, termed Iκb. Upon activation proteases digest phosphorylated IκB, releasing NF-κB dimers, which translocates to the nucleus. Binding to specific sequences in a diverse array of genes, induces mRNA transcription. Classes of genes induced by NF-κB include immuno-receptor genes (e.g. Igκ light chain, T-cell receptor-β chain, IL-2 receptor α-chain, MHC class I, β2-microglobulin), cytokine genes (e.g. β-interferon, GM-CSF, G-CSF, interleukin-6, interleukin-2, TNF-α), a proto-oncogene (*c-myc*), and viral genes (e.g. HIV LTR, cytomegalovirus immediate-early gene US 3) ((Lenardo *et al.*, 1987), (Jamieson *et al.*, 1989), (Leung and Nabel, 1988), (Baldwin Jr. and Sharp, 1988), (Visvanathan and Goodbourn, 1989), (Schreck and Baeuerle, 1990),(Lee *et al.*, 1995), [Nabel, 1987 #17], (Thrower *et al.*, 1996)). Modulation of gene transcription by NF-κB results in altered cell growth and function ((Miyamoto and Verma, 1995)). Furthermore, it has recently been demonstrated that NF-κB rescue from programmed cell death ((Wu *et al.*, 1996)), (Arsura *et al.*, 1996), (Schauer *et al.*, 1996)).

A number of stimuli, including ligated receptors such as Fas, TNF-α, CD40, immunoglobulin, antigen-specific T cell receptors, glucocorticoid receptor, and IL-1 cytokine receptors modulate NF-κB ((Miyamoto and Verma, 1995),(Verma *et al.*, 1995)). ((Olashaw *et al.*, 1992), (Auphan *et al.*, 1995),(Freimuth *et al.*, 1989), (Griffin *et al.*, 1989)). One common

element in NF-κB activation appears to be the induction of a state of oxidative stress (Schreck *et al.*, 1992). A recent report demonstating the ability of an environmental chemical, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), to activate a reporter gene linked to promoter sequences containing NF-κB binding sites (Yao *et al.*, 1995) suggests that pollutants can similarly activate NF-κB. An *in vivo* study demonstrating thymic NF-κB induction following exposure of rats to TCDD supports this hypothesis (Olnes *et al.*, 1994).

Our laboratory has investigated the aryl hydrocarbon receptor/transcription factor (AhR) which mediates most, if not all of the biologic activities of TCDD and related non-halogenated PAH ((Yamaguchi *et al.*, 1996), (Yamaguchi *et al.*, 1997), (Hardin *et al.*, 1992), (Cuthill *et al.*, 1991), (Safe, 1986), (Poland and Knutson, 1982)). The 8S AhR is stabilized by interaction with a 90 kD heat shock protein and is transformed into a transcription factor after ligand binding ((Denis *et al.*, 1988), (Pongratz *et al.*, 1992)). Ligand-bound AhR forms a dimer with an accessory molecule termed the aryl hydrocarbon receptor translocator protein (ARNT) through helix-loop-helix motifs present in both monomers (Reyes *et al.*, 1992). AhR-ARNT dimers bind specific DNA sequences, termed xenobiotic responsive elements (XRE). Included in the list of genes whose promotors contain XRE are proto-oncogenes (c-*myc*) (Lee *et al.*, 1995), cytokine genes (e.g. *IL-1β, TGF-β*) and genes encoding PAH metabolizing cytochrome P-450 enzymes (e.g. *CYP1A1* and *1A2*) ((Lyte and Bick, 1986), (Lusska *et al.*, 1993), (Cuthill *et al.*, 1991)). Although endogenous AhR ligands have not been defined, the array of genes apparantly affected by AhR engagement and the developmental defects manifest in AhR^{null} mice ((Schmidt *et al.*, 1996), (Fernandez-Salguero *et al.*, 1995)), suggest that the AhR plays a role in cell growth,.

Little is known about AhR interactions with other transcription factors (Sadek and Allen-Hoffmann, 1994). Significantly, many cellular changes effected by AhR activation, such as altered cytokine production, immunosuppression ((Faith and Moore, 1977), (Faith *et al.*, 1978), (Vos and Moore, 1974)), and the induction of programmed cell death ((Gao *et al.*, 1996), (Burchiel *et al.*, 1993), (Yamaguchi *et al.*, 1996) (Yamaguchi *et al.*, 1997)) are reminiscent of NF-κB-mediated cellular events. Given these observations, the functional similarity of the AhR to NF-κB modulating steroid receptors (Auphan *et al.*, 1995), and the likelihood that AhR regulated cytochrome P-450 activity pre-disposes cells to a state of oxidative stress, we hypothesized that AhR activation by PAH would result in NF-κB induction. To test this hypothesis, Hepa-1c1c7 (Hepa-1) liver cell lines expressing high AhR levels (Hankinson, 1983) and capable of supporting preB cells which undergo apoptosis on exposure to PAH (R. Near *et al*, submitted) were exposed to B[*a*]P or DMBA, and induction of NF-κB assessed. Hepa-1 mutants defective in AhR, ARNT or AhR-regulated P-4501A1 were exploited to address the role of the AhR and P-4501A1 enzymes in the putative induction of NF-κB. Results presented here support the hypothesis that PAH induce NF-κB through the AhR and suggest many of the biologic responses to PAH are mediated by the AhR and NF-κB.

Materials and Methods

Cell culture conditions: Wild type and mutant Hepa-1 cells were grown at 37° C, 10% CO_2 in DMEM supplemented with 10% bovine fetal serum (GIBCO-BRL Life Technologies, Inc. Grand Island, NY), 100 units/ml penicillin and streptomycin, 1mM glutamine, and 10^{-5} M β-mercaptoethanol. Cells were split 1:8 three times a week. To assess NF-κB activation, cultures were treated for one hour with vehicle (acetone) or 10^{-5}-10^{-8} M B[α]P or DMBA (Sigma, St.

Louis, MA) disolved in acetone (final vehicle concentration was 0.1%) in duplicate flasks. Vehicle had no effect on cultures. Similar results were obtained with all lines tested.

Cytosolic protein: Hepa-1 monolayers (approximately 5 x 10^6 cells) were released from culture flasks by treating for 3 min. with 0.25% trypsin/1mM EDTA*4Na (Life Technologies, Inc. Grand Island, NY). Cells were washed, re-suspended in lysis buffer (1% Triton X-100,150 mM NaCl, 25 mM Tris/HCl, 1 ug/ml aprotenin, 10 ug/ml leupeptin, 1 mM EDTA, 50 mM NaF, 1 mM orthovanadate, 1 mM PMSF) and nuclei removed by micro-centrifugation for 15 seconds at 15,000 x g. Supernatants containing cytosolic proteins were frozen at -20° C until use.

Nuclear protein isolation: Cell monolayers were lifted and washed as described above. Cells were pelleted, washed, and resuspended in 1 ml $P_{10}EG$ buffer (10% glycerol, KH_2PO_4*$3H_2O$ 5.7 mM, EDTA 7.5mM, pH 7.4) containing 0.2% Triton X-100 for Hepa-1 cells. Cell suspensions were rigorously pipetted. Nuclei were centrifuged for 12 min at 6,000 x G. $P_{10}EGD$ buffer/Triton X-100 treatment was repeated until microscopy confirmed a pure preparation of nuclei. Pellets of nuclei were washed twice with $P_{10}EGD$, resuspended in nuclei buffer (20 mM Hepes pH 7.9, 210 mM NaCl, 0.75 mM $MgCl_2$, 0.1 mM EDTA, 20% glycerol, 0.5 mM DTT and 0.5 mM PMSF added immediately before use) and microcentrifuged for 15 minutes at 15,000 x g. Cytosols were frozen at 20° C until use.

Western blotting: Cell suspensions were washed twice in cold PBS, resuspended in lysis buffer and centrifuged for 15 min at 15,000 x g. Supernatant protein concentrations 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% polyacrylamide SDS gels. Electrophoresis was carried out at 150 volts for 1 hour. Proteins were transferred to nitrocellulose filters (Bio-Rad Hercules, CA) at 150 milliamps for 1 hour or at 30 milliamps 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% nonfat dry milk, washed twice for 5 min in TBST, and incubated with monoclonal anti-AhR antibody Rpt1 ([Singh, 1993]) at a 1:10,000 dilution for 1 hour at room temperature. Filters were washed with TBST and incubated for 1 hour at room temperature with a 1:6,000 dilution of HRP-goat anti-mouse antibody (Sigma). Filters were washed twice and developed by chemiluminescence (Du Pont NEN Research Products Co., Boston, MA).

Electrophoretic gel mobility shift assay (EMSA): EMSA was used to assess binding of constitutive or activated NF-κB to a ^{32}P-labeled, 84 base pair DNA probe containing the 5'-GGGACTTTCC-3' sequence from the Igκ light chain enhancer. The pUC13 plasmid containing the NF-κB oligonucleotide was kindly provided by S.-T. Ju and T. L. Rothstein (Boston University). End-labeled DNA probes were generated by Klenow fill-in of 5' overhangs with ^{32}P-dATP. The reaction mix (2.5 µl 1 M Tris pH7.6, 2.5 µl 1M $MgCl_2$, 5 µl of 6000 Ci/mM ^{32}P-dNTPs, 1 µl Klenow enzyme, 500 ng of oligonucleotide probe, and double distilled water to bring the total volume to 26 µl) was incubated for 30 min. at 37° C. Labeled DNA was purified with Centri-Spin-20 columns (Princeton Separations, Adelphia, NJ) according to the manufacturer's instructions. Specific activity was determined and the volume adjusted with TE buffer to a final activity of 5,000 cpm/µl.

Nuclear protein (1 µg) was mixed with nuclei buffer to make a total volume of 5 µl, the volume of ^{32}P-labeled DNA probe containing 10,000 Cerenkov cpm, and the volume of double distilled water to bring the total mixture volume to 15 µl. The mixture was incubated at room

temperature for 20 min., and run 1 hour at 100 V in a 5% polyacrylamide non-denaturing gel. The gel was dried, and put on x-ray film with intensifying screens for overnight exposure at -80° C.

EMSA specificity was confirmed by addition of a 100-fold excess unlabelled NF-κB probe or irrelevant 32 base pair oligonucleotide 5'-TTTTTGAGCTCGGAGTTGCGTGAGAAGAGCCGGAGCCGGATC-3' containing the AhR XRE (Elferink *et al.*, 1990).

Annealing of the sense and anti-sense oligonucleotides to make AhR probe was performed by mixing of equal volumes of the sense- and anti-sense oligonucleotides in TE buffer (Tris 10mM, pH 7.6; EDTA 1mM pH 8.0), incubating the mix at 95° C for 10 min., and slowly cooling down to room temperature. DNA concentration was adjusted to 1 mg/ml.

To identify specific NF-κB/Rel subunits, nuclear extracts were incubated with ^{32}P-labeled DNA probe in the presence of 1 μg non-specific rabbit IgG as a negative control or 1 μg anti-p50/p105, p52/p100, c-Rel, or p65 (Rel A) antibody for 1 hour at room temperature. Samples were electrophoresed as described for the standard EMSA. All antibodies were produced by Santa Cruz Biotechnology, Santa Cruz, CA. These antibodies have been used extensively to characterize NF-κB/Rel family subunits (Lee *et al.*, 1995).

Ethoxyresorufin-o-deethylase (EROD) activity assay: Cytochrome P-4501A1 enzymes specifically convert the substrate 7-ethoxyresorufin to resorufin, resulting in a photometrically measurable color change. This EROD activity was assessed as previously described (Hahn and al., 1993). Briefly, cells were cultured in 48-well plates and treated for the specified amount of time with a specific PAH. Shortly before the assay, cells were rinsed with cold PBS to remove media. A reaction mixture was made so the final reaction contained 1.0 mM NADPH, 10μM dicumarol, 1.5mM salicylamide, and 2uM 7-ethoxyresorufin in a 50mM Tris-NaCl buffer. Immediately before reading, the reaction mixture was added to all wells except the blank well which received all reagents except substrate. Plates were read in a Cytofluor 2000 fluorimeter (Millipore, Bedford, MA) every 2 minutes for 20 minutes using an excitation wavelength of 530 nm and emission wavelength of 590 nm. Results were compared to a standard curve of resorufin in 50mM Tris-NaCl buffer. All chemicals for EROD assays were purchased from Sigma Chemical Co..

Results

Benzo[a]pyrene induces NF-κB in mouse hepatoma cells: To determine the potential for a prototypic PAH, B[*a*]P, to activate NF-κB in hepatoma cells, Hepa-1 cells were treated for 1 hour with vehicle, 10^{-5} or 10^{-4} M B[*a*]P. As a positive control, cells were treated with 10^{-3} M PMA. Cell nuclei were purified and nuclear protein tested for NF-κB oligonucleotide binding by EMSA. Data from a representative experiment are presented in Figure 1. A very low level of constitutive NF-κB binding was observed in vehicle-treated (Figure 1A) or untreated Hepa-1 cells (not shown). However, the NF-κB signal was significantly elevated in cells treated with B[*a*]P. It was generally noted that 10^{-4} M B[*a*]P induced lower levels of NF-κB binding than 10^{-5} M B[*a*]P and that, on avearge, NF-κB binding in response to 10^{-5} M B[*a*]P was approximately two fold less than that induced with PMA. These data suggest that, in mouse hepatoma cells, B[*a*]P exposure induces binding of NF-κB to the 84-bp oligonucleotide derived from the Igκ light chain gene promoter containing the core NF-κB binding site.

To confirm that NF-κB oligonucleotide binding was specific, Hepa-1 cells were treated with 10^{-6} - 10^{-8} M B[*a*]P, nuclear protein isolated, and EMSA performed in the absence or presence of 100 fold excess unlabelled NF-κB binding oligonucleotide or of an irrelevant oligonucleotide (XRE). Results from a representative experiment depicted in Figure 1B demonstrate significant NF-κB induction at doses as low as 10^{-8} M. An excess of cold NF-κB probe, but not XRE, prevented NF-κB binding. These results are consistent with the conclusion that the EMSA signal observed is due to specific binding of NF-κB oligonucleotide by members of the NF-κB/Rel family.

Benzo[a]pyrene- induced NF-κB binding protein contains p50 and p65 subunits : To evaluate which NF-κB/Rel family members (Miyamoto and Verma, 1995) are induced with B[*a*]P, supershift analyses of NF-κB were performed using antibodies specific for c-Rel, p52, p65, or p50. As previously demonstrated, addition of 10^{-6} M B[*a*]P to Hepa-1 cells induced significant NF-κB binding activity (Figure 2A). Rabbit IgG antibodies or antibodies specific for c-Rel or p52 did not alter the gel shift pattern. Significantly, antibodies specific for p65 or p50 significantly altered the B[*a*]P-induced banding pattern in Hepa-1 cells (Figure 2A; arrows). Appearance of a slower migrating supershifted band in the samples incubated with anti-p50 or anti-p65 antibodies suggests that the transcriptionally active p50/p65 heterodimer is present in B[*a*]P-treated cells. The possible presence of some p50/p50 or p65/65 homodimers cannot be ruled out. In control experiments, antibodies specific for p50, p65, and p52, but not c-Rel, supershifted NF-κB bands induced in WEHI-231 B cells with PMA (Figure 2B). These data are consistent with those previously reported (Lee *et al.*, 1995).

7,12 Dimethylbenz[a]anthracene (DMBA) induces NF-κB in mouse hepatoma cells: To determine if NF-κB activation is specific to B[*a*]P or can be extrapolated to another carcinogenic (RamaKrishna *et al.*, 1992) and immunosuppressive (Thurmond *et al.*, 1988) PAH/AhR ligand, Hepa-1 cells were treated for 1 hour with vehicle (acetone), 10^{-5} or 10^{-6} M DMBA. Nuclear proteins were extracted and NF-κB binding activity evaluated by EMSA. Both DMBA doses induced significant NF-κB binding (Figure 3) suggesting that NF-κB activation in mouse hepatoma cells may be generalized to another AhR-binding PAH.

Role of the AhR in B[a]P -induced NF-κB activation: To evaluate the possibility that B[*a*]P-induced NF-κB activation is an AhR-dependent process, a known inhibitor of AhR activity, α-naphthoflavone (ANF) ((Gaseiwicz and Rucci, 1991), (Kawabata and White Jr., 1987)), was tested for its ability to prevent NF-κB induction by B[*a*]P. ANF has been shown to competitively bind the AhR, prevent it's translocation to the nucleus, and to inhibit AhR-regulated cytochrome P-4501A1 activity ((Wilhemsson *et al.*, 1994), (Blank *et al.*, 1987), (Merchant *et al.*, 1990)). Treatment of Hepa-1 cells with 10^{-6} M B[*a*]P for 1 hour, but not with 10^{-5}-10^{-6} M ANF for 1-2 hours, induced NF-κB binding activity (Figure 4). Significantly, pretreatment for 1 hour with 10^{-6} or 5 x 10^{-6} M ANF completely blocked the B[*a*]P-induced NF-κB signal. These results are consistent with a role for the AhR and/or AhR-controlled P-450 enzyme activity in B[*a*]P-induced, NF-κB activation in mouse hepatoma cells.

B[*e*]P is a congener of B[*a*]P which weakly binds the AhR and is proportionately less bioactive ((White Jr and Holsapple, 1984), (Wojdani and Alfred, 1984), (White Jr. *et al.*, 1985), (Yamaguchi *et al.*, 1997)). If activation of the AhR is critical to the induction of NF-κB in this system, it would be predicted that B[*e*]P would not effect NF-κB activation. Indeed, while

significant NF-κB activity was induced with B[*a*]P, no activity was induced with 10^{-5} to 10^{-7} M B[*e*]P (Figure 5). Again, these results are consistent with a role for the AhR in NF-κB induction.

As a third approach to investigating the possible role of the AhR in B[*a*]P-induced NF-κB activation, the ability of B[*a*]P to induce NF-κB activity in mutant hepatoma cell lines deficient in AhR expression or signaling was tested. As previously reported (Miller *et al.*, 1983), Hepa-1 cells and the ARNT-defective Hepa-1 mutant BP^{r}C1 express AhR protein (Figure 6A). However, unlike wildtype Hepa-1 cells, Hepa-1 mutant BP^{r}C1 cells do not translocate AhR to the nucleus following exposure to AhR ligands (Figure 6B) (Miller *et al.*, 1983). Consequently, BP^{r}C1 cells fail to induce cytochrome P-4501A1 activity in response to PAH exposure (K. Mann *et al*, submitted). An additional hepatoma mutant, termed Hepa-1C2 (Hankinson, 1994), does not express AhR protein (Figure 6A). In this series of experiments, NF-κB binding activity was readily induced in wildtype Hepa-1 cells with B[*a*]P or PMA (Figure 7A). However, B[*a*]P did not induce NF-κB activity in either BP^{r}C1 (Figure 7B) or Hepa-1C2 (Figure 7C) cells. Similar results were obtained in 2 additional independent experiments. Both mutant cells lines appeared capable of inducing NF-κB binding activity in that both induced significant responses to PMA and/or H_2O_2 (Figures 7B and 7C). Although differences other than AhR signaling in these cell lines cannot be formally ruled out, these results suggest that a cell has to have functional AhR in order to respond to B[*a*]P treatment with NF-κB activation.

***Benzo[a]pyrene-induced NF-κB activation is independent of cytochrome P-450IA1 enzyme activity*:** Cytochrome P-450IA1 is one of the predominant enzymes induced following AhR-ligand binding ((Nebert *et al.*, 1990), (Lusska *et al.*, 1993)). P-450 baseline levels may be influenced by the level of constitutive AhR expression (Schmidt *et al.*, 1996). Therefore, the ability of ANF, an inhibitor of both AhR and P-450 activity, to block B[*a*]P induction of NF-κB and the failure to detect NF-κB induction with B[*a*]P in mutant cells lines, could reflect AhR and/or cytochrome P-450 dependent signaling. A hepatoma cell line defective in the expression of the P-450 encoding *Cyp1A1* gene due to missense mutations, Hepa-1C37 (Hankinson *et al.*, 1985), was used to address these possibilities. While this cell line expresses AhR (Figure 8A), it is incapable of inducing P-4501A1 enzyme activity, as assessed by the ethoxyresorufin deaminase (EROD) assay (Hahn and al., 1993), in response to AhR ligands, including the high affinity AhR ligand, TCDD (Figure 8B). Nevertheless, both B[*a*]P, the test PAH, and PMA, the control NF-κB activator, induced NF-κB in Hepa-1C37 cells (Figure 9). These results suggest that cytochrome P-4501A1 activity is not needed for NF-κB activation by B[*a*]P.

Discussion

In the present work, the possibility that activation of the aryl hydrocarbon receptor/transcription factor activates the NF-κB transcription factor was assessed. Using a well-defined mouse hepatoma cell line, Hepa-1, it was clearly demonstrated that a brief exposure of Hepa-1 cells to B[*a*]P or DMBA, prototypic PAH and AhR ligands, results in production of a nuclear factor capable of shifting the eletrophoretic mobility pattern of an oligonucleotide containing the NF-κB binding site from the Igκ light chain promotor. That this factor was authentic NF-κB was supported by the demonstration that: 1) the gel shift pattern induced with B[*a*]P was indistinguishable from that induced with PMA, a known NF-κB activator (Figures 1A and 7A), 2) unlabelled oligonucleotide containing the NF-κB site, but not an unrelated oligonucleotide (XRE; Figure 1B) or an oligonucleotide mutated in the NF-κB binding site (data

not shown), abrogated the B[*a*]P-induced band in EMSAs (Figure 1B), and 3) antibodies to NF-κB/Rel family members p50 and p65 supershifted the B[*a*]P-induced band in EMSAs (Figure 2A).

Since p50/p65 heterodimers are highly active in inducing gene transcription ((Miyamoto and Verma, 1995), (Baeuerle and Henkel, 1994)), the results suggest that B[*a*]P, and related xenobiotics, may inappropriately alter expression of a number of NF-κB-controlled genes which do not possess AhR binding sites. The resulting physiologic and pathologic processes could include modulation of cytokine production shown to occur after AhR-ligand exposure but through mechanisms that have not been completely defined (Moos *et al.*, 1997). In turn, this cytokine dysregulation could contribute to the myriad of immunosuppressive effects attributed to AhR ligands (Holsapple *et al.*, 1996). Similarly, PAH activation of NF-κB could result in augmented replication of viruses whose genomes contain NF-κB binding sequences ([Nabel, 1987 #17], (Thrower *et al.*, 1996)). Indeed, this possibilitiy has been suggested following the demonstration that cigarette smoking potentiates AIDS progression (Neiman *et al.*, 1993) and by the recent report that TCDD induces transcription of a chloramphenicol acetyltransferase (CAT) reporter gene construct driven by the HIV-LTR in part through it's NF-κB binding site (Yao *et al.*, 1995). In considering possible *in vivo* effects of PAH exposure, it is important to note that a relatively low concentration of B[*a*]P (i.e. 10^{-8} M) induced NF-κB activation in the present system. This dose is three logs lower than that reported to induce transcription of the HIV-LTR-driven reporter construct (Yao *et al.*, 1995) and approaches a physiologically relevant dose. The effects of yet lower PAH doses on NF-κB after longer term exposure is under investigation.

With regard to the mechanism through which B[*a*]P and, presumably DMBA, induces NF-κB, three lines of evidence point to a role for the AhR. First, while incapable of inducing NF-κB activity itself, α-naphthoflavone blocked NF-κB induction with B[*a*]P. ANF inhibits AhR translocation (data not shown)((Gaseiwicz and Rucci, 1991)) and blocks AhR-regulated cytochrome P-450 activity. Secondly, B[*e*]P, a stereoisomer of B[*a*]P which does not activate the AhR, failed to induce NF-κB activity. Third, Hepa-1 mutants defective either in AhR expression (Hepa-1C2) or AhR complex function (BP^{r}C1) did not activate NF-κB following B[*a*]P exposure. Wildtype Hepa-1 and both Hepa-1 mutants were capable of NF-κB activation in response to PMA treatment, demonstrating that the molecular machinery required for NF-κB activation is intact in all cell lines tested.

The means through which the AhR induces NF-κB is not yet clear. One possibility is that activation of the AhR induces cytochrome P-450 which in turn oxidizes parent compounds, generating reactive oxygen intermediates. A resulting state of oxidative stress could activate NF-κB (Schreck *et al.*, 1992). This scenario is easily envisioned with readily metabolized substrates such as B[*a*]P and DMBA. Oxidative stress produced by PAH metabolites could be exacerbated by arachidonic acid metabolites generated through PAH-induced P-450 enzyme activity (Rifkind *et al.*, 1990). In either case, the activity of P-4501A1 would not be predicted to be critical to PAH-induced NF-κB induction since NF-κB activity was readily induced in Hepa-1c37, a Hepa-1 mutant defective in P-450 1A1 activity. This result may be contrasted with that of Yao *et al* who reported that TCDD could not induce transcription of the HIV-LTR-driven reporter construct in Hepa-1c37 cells 24 hours after treatment (Yao *et al.*, 1995). This difference in the two systems, together with the reported failure of DMBA to induce HIV-LTR-CAT activity (Yao *et al.*, 1995), could be attributed to differential NF-κB regulation of HIV-LTR and Igκ light chain promotor

regions. In this regard, the identities of the NF-κB/Rel subunits postulated to be involved in TCDD-induced HIV-LTR-CAT activity were not determined (Yao *et al.*, 1995).

Finally, these studies were motivated in part by the observation that preB cells maintained on bone marrow stromal cells ((Yamaguchi *et al.*, 1997), (Hardin *et al.*, 1992)) or Hepa-1 monolayers (R. Near *et al*, submitted) undergo apoptosis when exposed to B[*a*]P or DMBA. In these systems, which model B lymphopoiesis in the bone marrow and fetal liver respectively, PAH-treated, AhR^+ supporting stromal cells deliver a death signal to AhR^- preB cells. Like PAH-induced NF-κB activation, induction of the death signal in stromal cells is AhR- but not P450 IA1- dependent. The possibility that AhR-dependent, NF-κB activation in lymphopoietic microenvironments exposed to low PAH doses results in immature lymphocyte death must be considered given the observations that both B[*a*]P and DMBA are capable of inducing NF-κB and preB cell apoptosis. Experiments to address the putative connection between AhR-dependent NF-κB activation and immature lymphocyte apoptosis are underway.

Legends to Figures:

Figure 1. *B[a]P and PMA activate NF-κB in wildtype Hepa-1 cells:* Hepa-1 cells were treated for 1 hour with 10^{-9} M PMA, 10^{-5}-10^{-8} M B[*a*]P or vehicle. **Panel A:** Nuclear protein was extracted and NF-κB binding activity evaluated by EMSA. Where indicated (**Panel B**) EMSA was performed in the presence or absence of 300 ng of unlabelled NF-κB binding oligonucleotide (" cold NF-κB") or an irrelevant oligonucleotide ("cold XRE"). Results representative of three experiments are presented.

Figure 2. *Supershift analyses of NF-κB/Rel family members induced by B[a]P:* **Panel A:** Nuclear protein isolated from Hepa-1 cells treated for 1 hour with 10^{-6} M B[*a*]P was incubated with ^{32}P-labeled DNA probe in the presence of 1 μg non-specific rabbit IgG as a negative control or 1 μg anti-p50, -p52, -c-Rel, or -p65 (Rel A) antibody for 1 hour at room temperature. Samples were electrophoresed as described for the standard EMSA. **Panel B:** Nuclear protein isolated from 10^{-9} M PMA-treated WEHI-231 cells were incubated with ^{32}P-labeled DNA probe in the presence of 1 μg non-specific rabbit IgG or anti-p50, -p52, -c-Rel, or -p65 (Rel A) antibody and electrophoresed as described above.

Figure 3. *DMBA induces NF-κB in mouse hepatoma cells:* Hepa-1 cells were treated for 1 hour with vehicle, 10^{-5} or 10^{-6} M DMBA, nuclear protein extracted and NF-κB binding activity evaluated by EMSA.

Figure 4. *ANF pretreatment prevents B[α]P-induced NF-κB activation:* Hepa-1 cells were pretreated for 1 hour with 10^{-6}, 5 x 10^{-6} or 10^{-5} M ANF, or acetone. Pretreatment was followed by a 1 hour incubation with 10^{-6} M B[*a*]P or acetone. Nuclear protein was isolated and NF-κB EMSA performed.

Figure 5. *B[e]P does not induce NF-κB in mouse hepatoma cells:* Hepa-1 cells were treated for 1 hour with 10^{-5} M B[α]P, vehicle, 10^{-5} or 10^{-6} M B[*e*]P, nuclear protein extracted and NF-κB binding activity evaluated by EMSA.

Figure 6. *Characterization of Hepa-1 AhR and ARNT mutants:* **Panel A:** Hepa-1, BP^{r}C1 and Hepa-1C2 cells were lysed and cytosolic protein purified. Protein (15 μg) was administered to each well of 7.5% SDS polyacrylamide gel to be separated by Laemmli electrophoresis, transferred to nitrocellulose, and tested for the presence of AhR by Western blotting with monoclonal anti-AhR antibody Rpt1. **Panel B:** Nuclei were isolated from Hepa-1 and BP^{r}C1 cells treated with acetone or B[*a*]P for 45 minutes. Nuclear protein was extracted and 15 μg of nuclear protein electrophoresed and analyzed by Western blotting as above. Figures represents the results of at least 3 independent experiments.

Figure 7. *B[a]P activates NF-κB in wildtype but not AhR complex mutant Hepa-1 cells:* Wildtype Hepa-1 cells (panel A), ARNT defective BP^{r}Cl (panel B), or AhR-defective C2 (panel C) cells were treated for 1 hour with B[*a*]P, vehicle, 10^{-9} M PMA, or 100 μM H_2O_2. Nuclear protein was extracted and NF-κB binding activity evaluated by EMSA.

Figure 8. *Characterization of CYPIA1-defective Hepa-1C37 cells:* **Panel A:** Hepa-1 and Hepa-1C37 cells were lysed, cytosolic protein extracted, and 15 μg electrophoresed and immunoblotted for AhR expression as described in Figure 6. **Panel B:** KOREN'S LEGEND. WHERE IS KOREN'S LEGEND?????

Figure 9 . *B[a]P and PMA activate NF-κB in Hepa-1C37 cells:* Hepa-1C37 cells were treated for 1 hour with acetone, 10^{-9} M PMA, or 10^{-5} M B[*a*]P, nuclear protein extracted and NF-κB binding activity evaluated by EMSA. The figure represents the results of two experiments with two independent duplicates run in each experiment.

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Abbreviations:

AhR: Aryl hydrocarbon receptor; B[*a*]P: benzo[*a*]pyrene; DMBA: 7,12-dimethylbenz[*a*]anthracene; PAH: polycyclic aromatic hydrocarbon(s); TCDD: 2,3,7,8 tetrachlorodibenzo-*p*-dioxin

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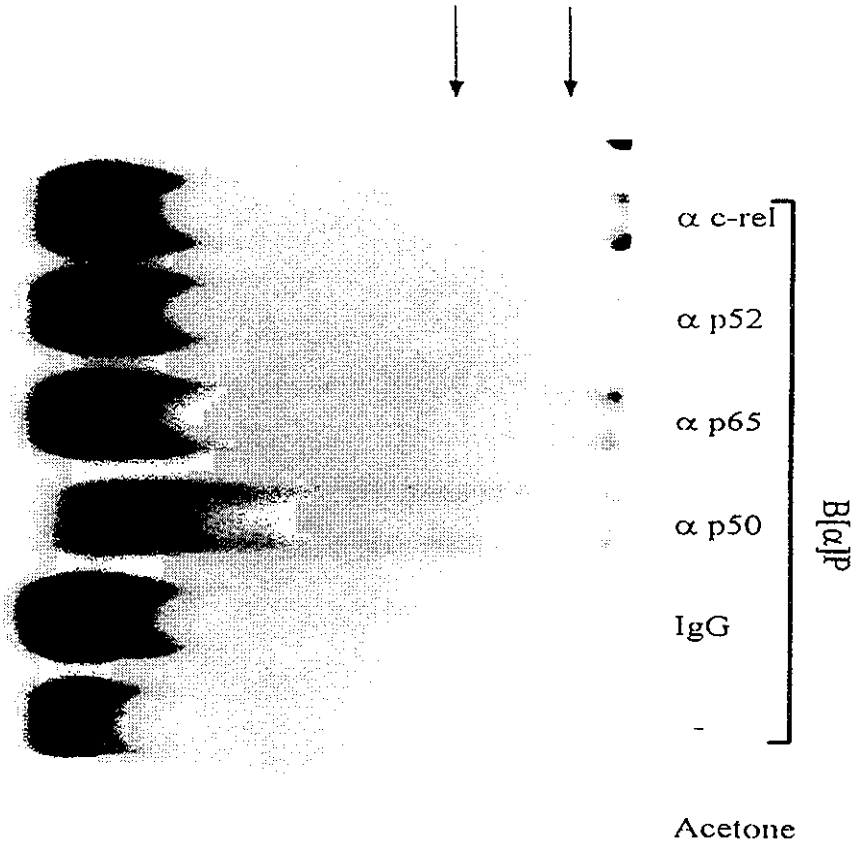
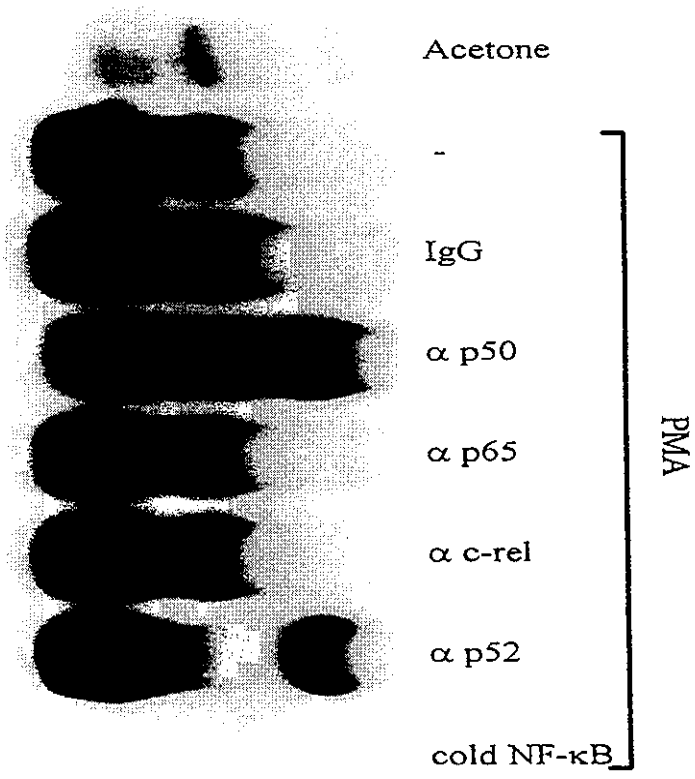


Figur 2A



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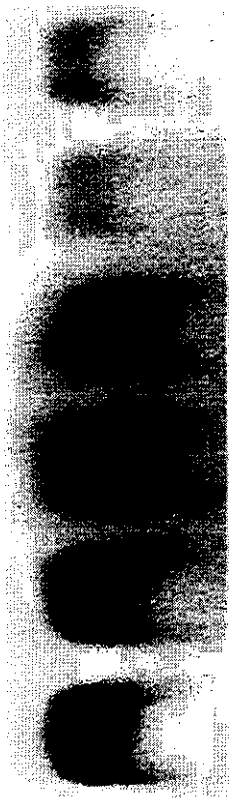
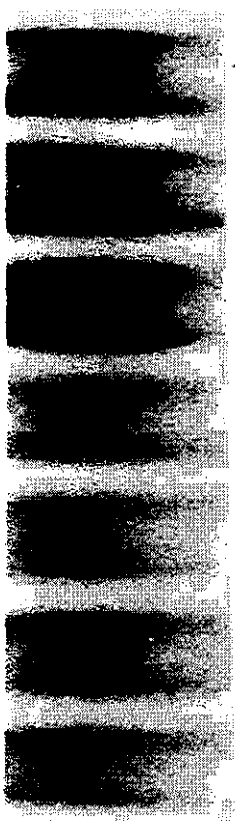


Acetone

10^{-5} M DMBA

10^{-6} M DMBA

Figur 3



Acetone

10^{-6} M ANF/Acetone

Acetone/10^{-6} M B [α]P

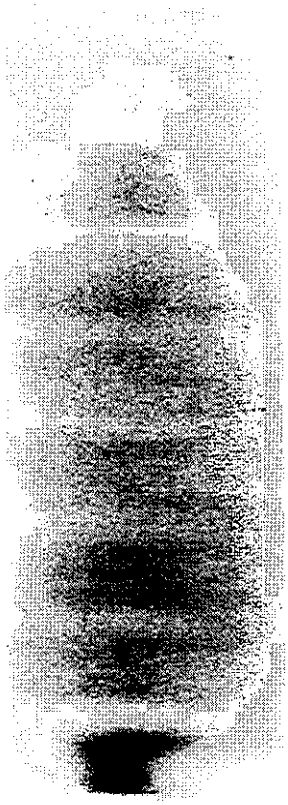
$5x10^{-6}$ M ANF/Acetone

10^{-5} M ANF/Acetone

10^{-6} M ANF/10^{-6} M B [α]P

$5x10^{-6}$ M ANF/10^{-6} M B [α]P

Figur 4



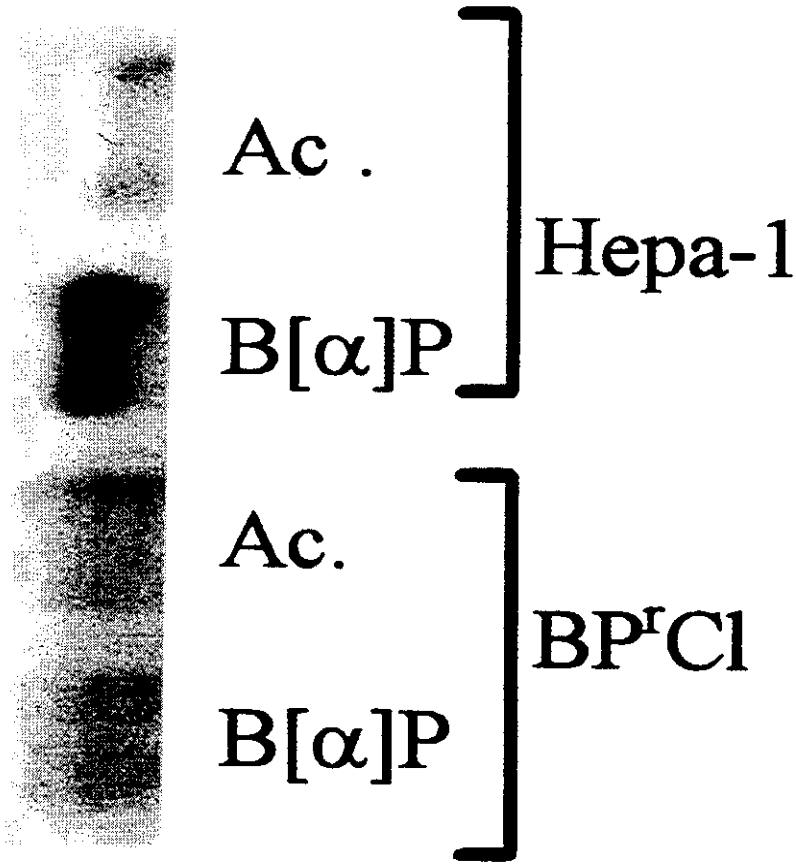
Figur 5

Hepa-1

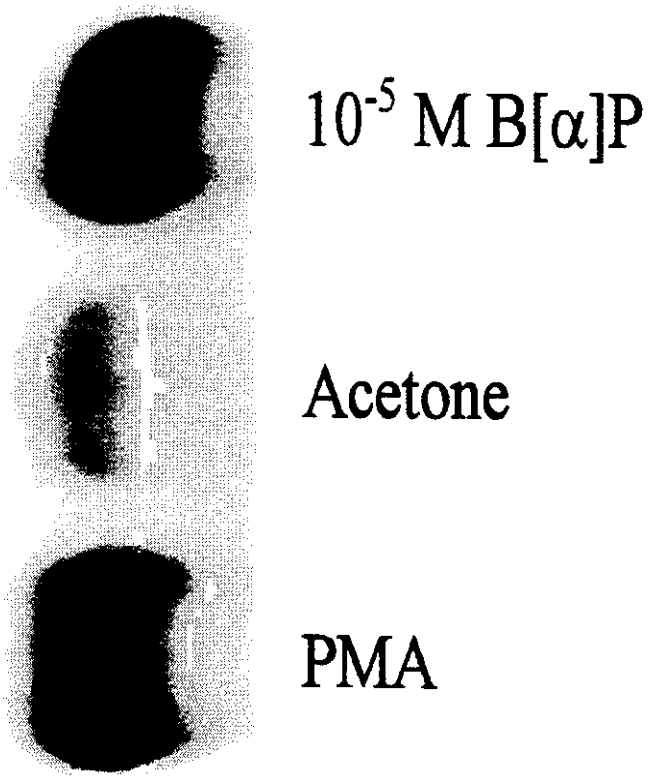
BP^rCl

C2

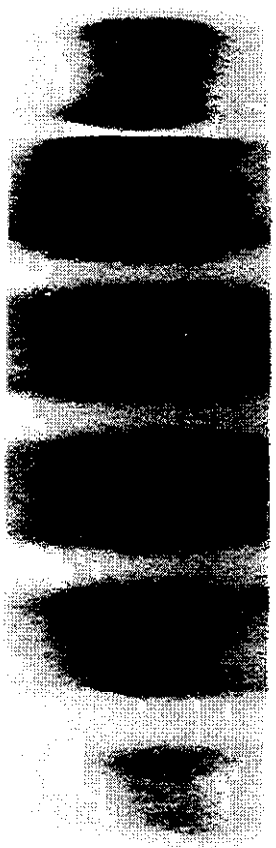
Figur 6A



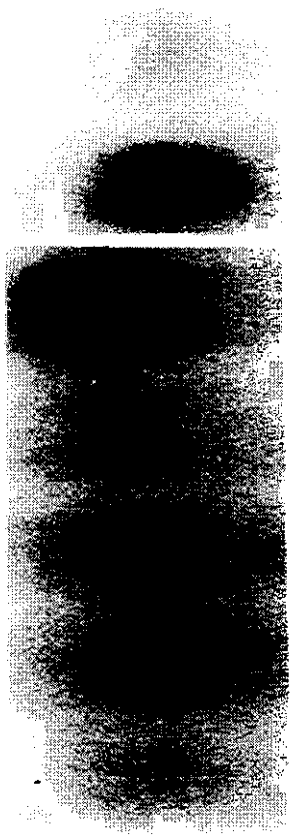
Figur 6B



Figur 7A



Figur 7B

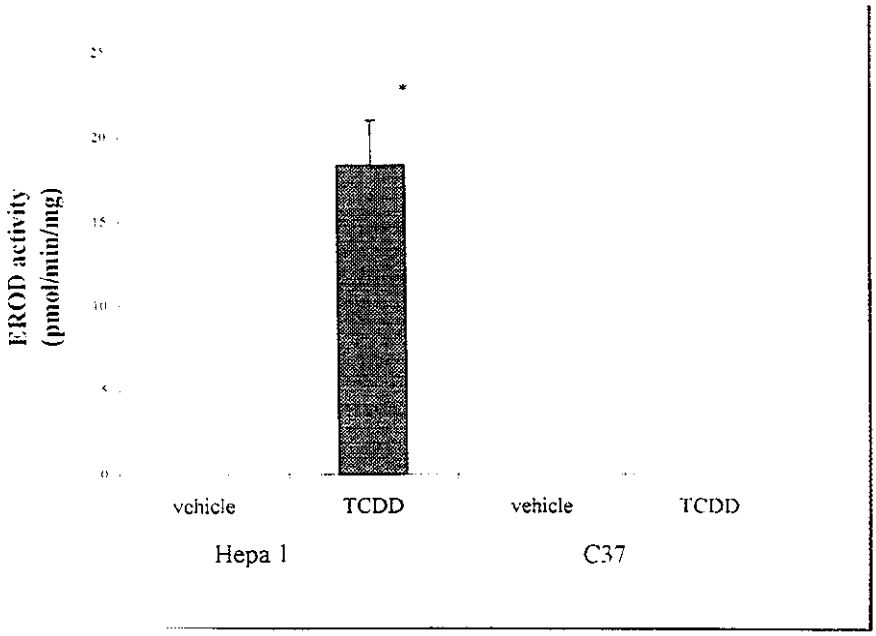


Figur 7C

Hepa-1

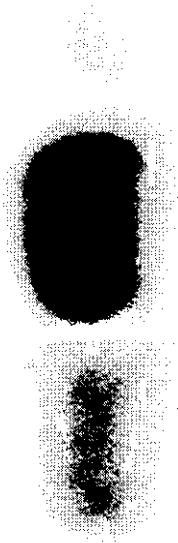
C37

Figur 8A



Figur 8B

Acetone



PMA

10^{-6} M B[α]P

Figur 8C

Article 5

Induction of PreB Cell Apoptosis by 7,12-Demithylbenz[*a*]anthracene in a Long-Term Primary Murine Bone Marrow Cultures.

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Induction of PreB Cell Apoptosis by 7,12-Dimethylbenz[a]anthracene in Long-Term Primary Murine Bone Marrow Cultures[1]

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Induction of PreB Cell Apoptosis by 7,12-Dimethylbenz[a]-anthracene in Long-Term Bone Marrow Cultures. Yamaguchi, K., Matulka, R. A., Shneider, A. M., Toselli, P., Trombino, A. F., Yang, S., Hafer, L. J., Mann, K. K., Tao, X.-J., Tilly, J. L., Near, R. I., and Sherr, D. H. (1997). *Toxicol. Appl. Pharmacol.* 147, 190–203.

Numerous studies demonstrate that polycyclic aromatic hydrocarbons (PAH) suppress immunity by modifying the function of both B and T cells. Relatively few studies have assessed the effects of these common environmental chemicals on immature lymphocytes. In the present study, long-term primary bone marrow cultures were employed to investigate the effects of a prototypic PAH and aryl hydrocarbon receptor (AhR) agonist, 7,12-dimethylbenz[a]anthracene (DMBA), on immature B lymphocytes. In this system, immature preB cells are maintained in a supportive microenvironment provided by bone marrow stromal cells. Results presented here demonstrate that (1) exposure of primary bone marrow cultures to DMBA results in preB cell death by apoptosis; (2) notably low doses of DMBA ($\geq 10^{-8}$ M) induce preB cell apoptosis; (3) in long-term cultures, bone marrow stromal cells, but not preB cells, express AhR mRNA and protein as determined by *in situ* hybridization, RT-PCR, and immunoblotting; (4) freshly isolated unfractionated bone marrow cells, but not purified bone marrow B cells, express AhR protein as assessed by immunohistochemistry; (5) α-naphthoflavone, a competitive AhR inhibitor and cytochrome P450 antagonist, completely blocks DMBA-induced preB cell apoptosis in primary bone marrow cultures; and (6) DMBA or benzo[a]pyrene injection *in vivo* results in bone marrow cell apoptosis consistent with the death of hematopoietic cells clustered around stromal elements. The results implicate programmed cell death as a mechanism underlying DMBA-mediated immunosuppression and suggest that preB cell death is influenced by local interactions with AhR^+ bone marrow stromal cells. © 1997 Academic Press

For many years research with polycyclic aromatic hydrocarbons (PAH)[2] has focused on the ability of these common environmental chemicals to induce cell transformation. More recently PAH have been investigated because of their more acute induction of a number of biologic responses including immunosuppression (Burchiel *et al.*, 1992, 1993; Davila *et al.*, 1995; Hardin *et al.*, 1992; Hinoshita *et al.*, 1992; Kawabata and White 1987; Thurmond *et al.*, 1988; White and Holsapple 1984; White *et al.*, 1985; Wojdani *et al.*, 1984; Yamaguchi *et al.*, 1996; Temple *et al.*, 1993). Several PAH, including 7,12-dimethylbenz[a]anthracene (DMBA), benzo[a]pyrene (B[a]P), and fluoranthene, as well as related halogenated hydrocarbons like 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and polychlorinated biphenyls (PCBs), induce thymic atrophy, decrease resistance to infectious agents and transplantable tumors, reduce bone marrow cellularity, alter lymphocyte homing, impair B and T lymphocyte proliferative responses, inhibit B cell antibody responses, decrease cytotoxic T cell activity, induce cell death in myeloid, B, and T cells, inhibit natural killer activity, or decrease cytokine production in animal model systems (Davis and Safe, 1991; Dooley and Holsapple 1988; Fine *et al.*, 1990; Gasiewicz and Rucci 1991; Greenlee *et al.*, 1985; Harper *et al.*, 1995; Holladay and Smith 1995; Holsapple *et al.*, 1991; Kerkvliet *et al.*, 1990; Kremer *et al.*, 1994; Morris *et al.*, 1994; Wood *et al.*, 1993; Ackerman *et al.*, 1989; Fine *et al.*, 1990; Kerkvliet *et al.*, 1990; Silkworth *et al.*, 1984; Burchiel *et al.*, 1992; White and Holsapple, 1984; Karras and Holsapple,1994). Epidemiological studies suggest that PAH are immunosuppressive in humans as well (Hoffman *et al.*, 1986; Kimbrough, 1987; Szczeklik *et al.*, 1994). Although the intracellular mechanisms by which PAH suppress immunity are ill-defined, some studies support a role for the aryl hydro-

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[2] Abbreviations used: AhR, aryl hydrocarbon receptor; B[a]P, benzo[a]-pyrene; DMBA, 7,12-dimethylbenz[a]anthracene; PAH, polycyclic aromatic hydrocarbon(s); PI, propidium iodide; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

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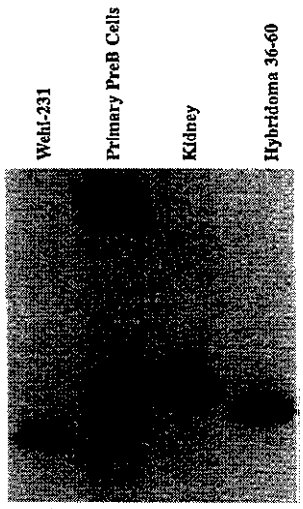


FIG. 1. Long-term bone marrow cultures contain preB cells. Lymphoid cells (>90% CD45/B220$^+$) were harvested from adherent monolayers of primary bone marrow stromal cells. DNA was extracted from these cells, WEHI-231 (B cell lymphoma) cells, kidney cells, and 36–60 B cell hybridoma cells, electrophoresed, and blotted with a JH probe. Germ-line IgH genes are evident in kidney cells. Rearranged IgH genes are present in clonal WEHI-231 and 36–60 hybridoma cells. A smear of rearranged IgH genes in B cells from bone marrow cultures is characteristic of a population of preB cells.

carbon receptor (AhR) and/or PAH metabolism in immunosuppression (Hardin *et al.*, 1992; Holsapple *et al.*, 1991; Kerkvliet *et al.*, 1990; Ladics *et al.*, 1991; Morris *et al.*, 1994; Silkworth *et al.*, 1984; Thurmond *et al.*, 1988; White *et al.*, 1985; Harper *et al.*, 1994).

Relatively few studies have evaluated the effects of PAH or related halogenated hydrocarbons on developing immune systems (Morris *et al.*, 1994; Blaylock *et al.*, 1992; Luster *et al.*, 1988), an important consideration given the comparative sensitivity of developing biologic systems to environmental pollutants (Holladay and Smith, 1995). In the present study, the ability of a prototypic PAH, DMBA, to alter lymphocyte growth in an *in vitro* model of B lymphopoiesis was studied. Our investigations were motivated by studies demonstrating DMBA-induced suppression of mature lymphocyte activity *in vivo* and *in vitro* (Holladay and Smith, 1995; Burchiel *et al.*, 1992, 1993; Davila *et al.*, 1995; Ladics *et al.*, 1991).

As *in vivo*, growth and development of bone marrow-derived preB cells in long-term bone marrow cultures (Whitlock *et al.*, 1984) are dependent on contact with, and growth factor production by, bone marrow stromal cells (Pietrangeli *et al.*, 1988). Thus, adverse effects of DMBA on preB cell populations could reflect direct effects on the preB cells themselves and/or stromal cells on which preB cells depend for growth support. In the present studies, particular emphasis was placed on identifying the mechanism(s) of DMBA immunotoxicity, the possible role of the AhR or PAH metabolism in biologic responses to DMBA, and the potential for DMBA immunotoxicity to be mediated by modification of bone marrow stromal cell function, rather than by direct effects on immature B lymphocytes.

MATERIALS AND METHODS

Bone marrow cultures. Murine bone marrow cultures were prepared from C57BL/6 (AhR^b) bone marrow as described (Whitlock *et al.*, 1984). Briefly, bone marrow was expunged from the femurs of 3- to 4-week-old mice, washed, counted, and cultured in RPMI medium containing 5% FCS (Gibco/BRL, Inc., Grand Island, NY), 2 mM *L*-glutamine (Gibco/BRL), 50 U/ml penicillin–streptomycin (Gibco/BRL), and 0.05 mM 2-mercaptoethanol (Mallinckrodt, Paris, KY) (3×10^6 cells/3 ml in 6-well plates). One-half of the medium was replaced every 3–4 days with fresh medium. Growth of stromal cells and stromal cell-adherent lymphocytes was routinely evident after 10 days. After 4 weeks of culture, >90% of stromal cell-adherent cells consisted of B220$^+$, sIgM$^-$ B lymphocytes as assessed by flow cytometry (see below) with rearranged immunoglobulin heavy chains (Fig. 1). 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. At various points thereafter preB cells from duplicate wells were pooled and assayed for apoptosis. Vehicle had no effect on cultures.

Southern blot analysis of Ig heavy chain gene rearrangements. Procedures for Southern blotting and hybridization using the JH probe "P2" have been previously described in detail (Near and Haber, 1989) with the exceptions that charged nylon filters were used and that the DNA transfer

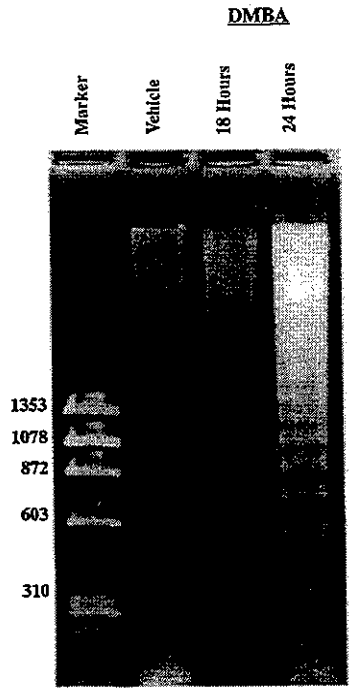


FIG. 2. DMBA induces DNA fragmentation in primary bone marrow cultures. Vehicle (0.1%) or DMBA (10^{-5} M) was added to 3- to 4-week-old bone marrow cultures in duplicate wells. Cells were harvested after 18 and 24 hr, DNA extracted, and electrophoresed in 3.5% agarose gels. Digestion of DNA into oligonucleosomal fragments (i.e., multiples of 200 base pairs) results in a ladder pattern characteristic of apoptosis.

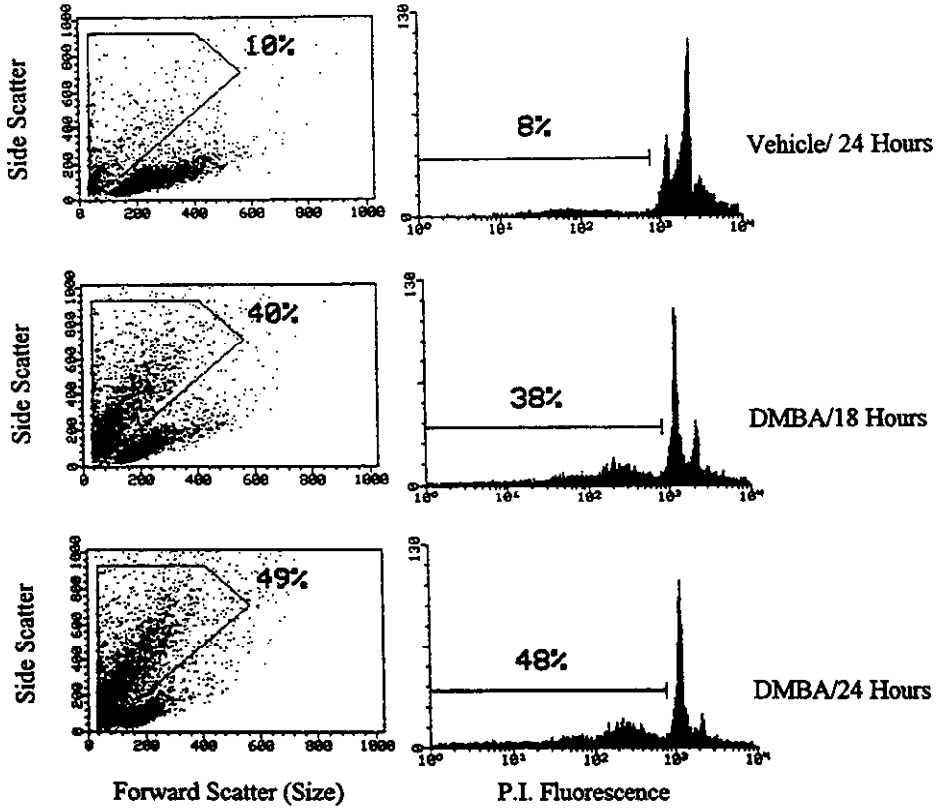


FIG. 3. DMBA induces morphologic and chromatin changes characteristic of apoptosis in primary preB cell populations. Vehicle (0.1%) or DMBA (10^{-4} M) was added to 3- to 4-week-old bone marrow cultures in duplicate wells. Cells were harvested after 18 and 24 hr. Cells were divided into two aliquots. Cells from one aliquot were treated with PI in hypotonic buffer for DNA analyses (histograms). Cells from the second aliquot were resuspended in PBS for forward (size) and side (granularity) light-scatter analyses (dot plots). Apoptotic cells stain poorly with PI (regions in histograms) and are relatively small and granular (enclosed region of each dot plot). A representative experiment is shown. Comparable results were obtained with lower doses of DMBA (see Fig. 4).

was done using alkaline transfer as described by the manufacturer (Gene Screen Plus, NEN Research Products, Boston, MA).

Fluorescence analyses and sorting. PreB cells were harvested by gently washing 3- to 4-week-old primary bone marrow cultures with media. To assess purity, preB cells were incubated for 40 min on ice with phycoerythrin-anti-CD45/B220 (clone RA3-6B2, rat IgG 2a, Pharmingen) or with a phycoerythrin-labeled isotype control. Cells were washed and analyzed in a Becton–Dickinson FACScan flow cytometer. To sort CD45/B220$^+$ preB cells from bone marrow cultures or to sort bone marrow B cells from freshly isolated bone marrow, 10^6 cells were incubated as above with PE-anti-CD45/B220 or PE-conjugated isotype control antibody, washed in medium containing 20% FCS, and sorted with a Becton–Dickinson FACStar on the basis of lymphocyte morphology (forward and side light-scatter parameters) and expression of CD45/B220. Sorted cells were reanalyzed after sorting and consisted of >95% CD45/B220$^+$ cells.

Quantitation of apoptotic cells was performed as previously described (Hardin *et al.*, 1992; Hinoshita *et al.*, 1992; Yamaguchi *et al.*, 1996). Cells were washed in cold PBS, pelleted, and resuspended in 0.5 ml of hypotonic fluorochrome solution containing 50 μg/ml propidium iodide (PI; Sigma Chemical Co., St. Louis, MO), 1% sodium citrate, and 0.1% Triton X-100 (Sigma). Cells exhibiting 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 by flow cytometry, cells were resuspended in PBS containing 10% FCS.

DNA gels. Cells (10^6) were washed and resuspended in cold 10 mM Tris/1 mM EDTA (TE) 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 with phenol–chloroform. Fragmented DNA in supernatants was precipitated with ethanol, pelleted, rinsed with cold ethanol, dried, and resuspended in Tris/EDTA buffer. For gel electrophoresis, samples were added to loading buffer consisting of 40% sucrose in Tris/EDTA buffer, 1% SDS (Sigma), bromophenol blue, and 2.5 μg/ml RNase (Gibco/BRL) 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 hr and stained with ethidium bromide.

AhR immunoblotting. PreB cells were gently washed off bone marrow stromal cell monolayers and transferred to new culture wells for 3 hr before harvest to minimize contamination with plate-adherent stromal cells. Stromal cells were lifted from plates by a 3-min treatment with 0.25% trypsin containing 1 mM EDTA·4Na (Gibco-BRL). Cells were washed twice in cold PBS, resuspended in lysing buffer (1% Triton X-100, 150 mM NaCl, 25 mM Tris–HCl, 1 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM EDTA, 50 mM NaF, 1 mM orthovanadate, 1 mM PMSF), and centrifuged for 15 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 diluted in Laemmli buffer and loaded into 7.5% SDS–polyacrylamide gels. Electrophoresis was carried out at 150 V for 1 hr. Proteins were transferred from gels to nitrocellulose filters (Bio-Rad, Her-

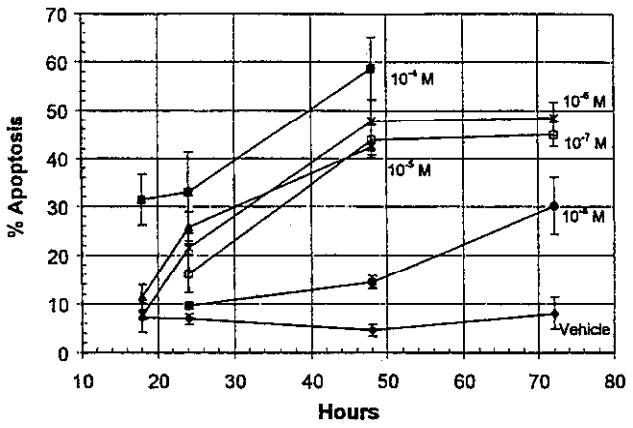


FIG. 4. Kinetics and dose response of primary preB cells to DMBA. Vehicle (0.1%) or DMBA (10^{-4}–10^{-8} M) was added to 3- to 4-week-old bone marrow cultures. Eighteen to 72 hr latter preB cells were harvested and stained with PI, and the percentage of cells undergoing apoptosis was quantitated by flow cytometry. Each data point represents data obtained from three to four experiments. Apoptosis following DMBA exposure reached statistical significance relative to vehicle controls ($p < 0.05$) at 18 hr with 10^{-4} M, at 24 hr with 10^{-6} M, and at 48 hr with 10^{-8} M DMBA.

cules, CA) at 150 V for 1 hr 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) (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 monoclonal anti-AhR antibody Rpt1 (Singh and Perdew, 1993) 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 1:6000 dilution of HRP-goat anti-mouse antibody (Sigma). Filters were washed twice and developed by chemiluminescence (Du Pont NEN Research Products Co., Boston, MA).

Immunohistochemistry. Freshly isolated bone marrow cells or CD45/B220$^+$ cells were cytospun onto glass microscope slides, air dried, and fixed in 10% formalin. Slides were then incubated with 2 μg/ml rabbit polyclonal anti-AhR antibodies or control rabbit immunoglobulin for 1 hr at 37°C and washed and incubated with a 1:300 dilution of biotinylated swine anti-rabbit immunoglobulin antibody for 30 min at 37°C. Background peroxidase activity was quenched with 3% H_2O_2 for 10 min, cells were washed, and horseradish peroxidase-conjugated streptavidin was added for a 25-min incubation at 37°C. AhR-specific staining was visualized by incubating slides in 0.1% 3,3'-diaminobenzidine and 0.04% H_2O_2 (DAKO, Carpinteria, CA) in PBS solution for 10 min at room temperature. Slides were then washed in running tap water and counterstained with hematoxylin, coverslipped with Permount, and examined by light microscope. AhR staining under these conditions was completely inhibited by absorbing anti-AhR antibody with Sepharose beads conjugated with recombinant AhR protein. The stain was not affected by absorbing anti-AhR antibody with Sepharose beads conjugated with an irrelevant protein, lysozyme (data not shown).

RT-PCR for AhR mRNA. PreB cells were washed off stromal cell monolayers by vigorous pipetting and RNA prepared from 5×10^6 cells as described (Leedo Medical Laboratories, Houston, TX). Adherent stromal cells ($3–5 \times 10^6$) were lysed directly in tissue culture wells. Integrity of RNA samples was assayed by electrophoresis in 1.5% agarose gels prior to RT-PCR to detect AhR mRNA in 5-μg samples (total RNA) as described (SuperScript Preamplification System; Gibco/BRL). cDNA was amplified for 35 cycles with $MgCl_2$ concentration adjusted to 2.5 mM to maximize specific signal and using the following AhR primers: CTGGCAATGAATTTCCAAGGGAGG and CTTTCTCCAGTCTTAATCATGCG. Primers were chosen to enclose the sequence which contains the putative murine AhR ligand-binding domain (Dolwick *et al.*, 1993; Ema *et al.*, 1992). Amplified DNA was electrophoresed through 3% gels (3:1 NuSieve:LE agarose; FMC, Rockland, ME) and DNA visualized by ethidium bromide staining. All samples were normalized for equal loading of gels with the GADPH housekeeping gene. GADPH mRNA was reverse transcribed and cDNA amplified with the following primers: CCATCACCATCTTCCAGGAG and CCTGCTTCACCACCTTCTTG.

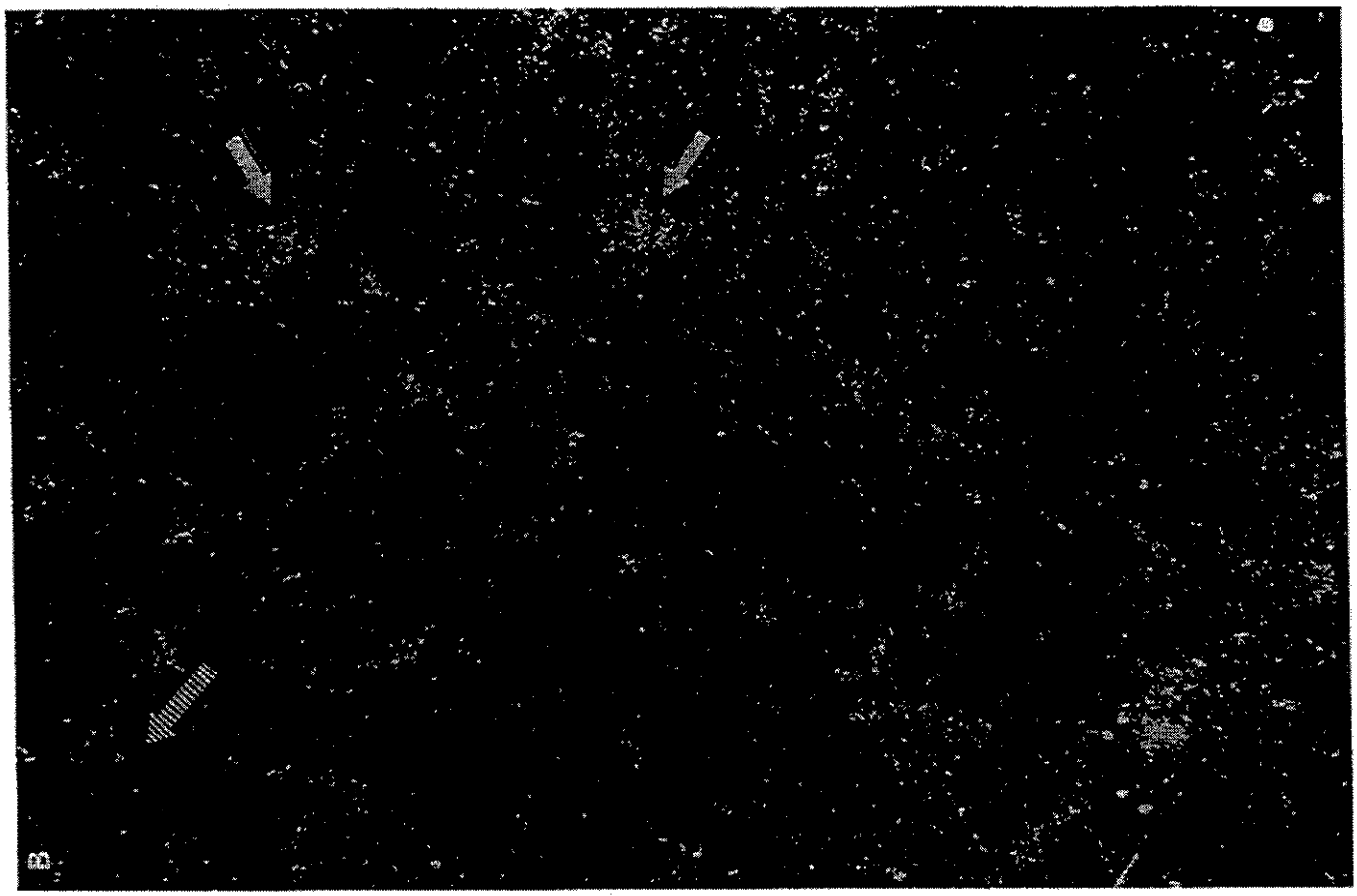
In situ hybridization. ^{35}S-radiolabeled AhR riboprobes were generated using T7 (sense) and SP6 (antisense) promoters with linearized *Xba*I and *Hin*dIII digests of pcDNA-AhR murine AhR cDNA as template. PreB cells harvested from 4-week-old bone marrow cultures were fixed with 4% paraformaldehyde, dehydrated, and hybridized for 18 hr at 52°C with either sense or antisense riboprobe. Slides were washed and autoradiographs exposed for 6 weeks. No signal was observed with the sense riboprobe.

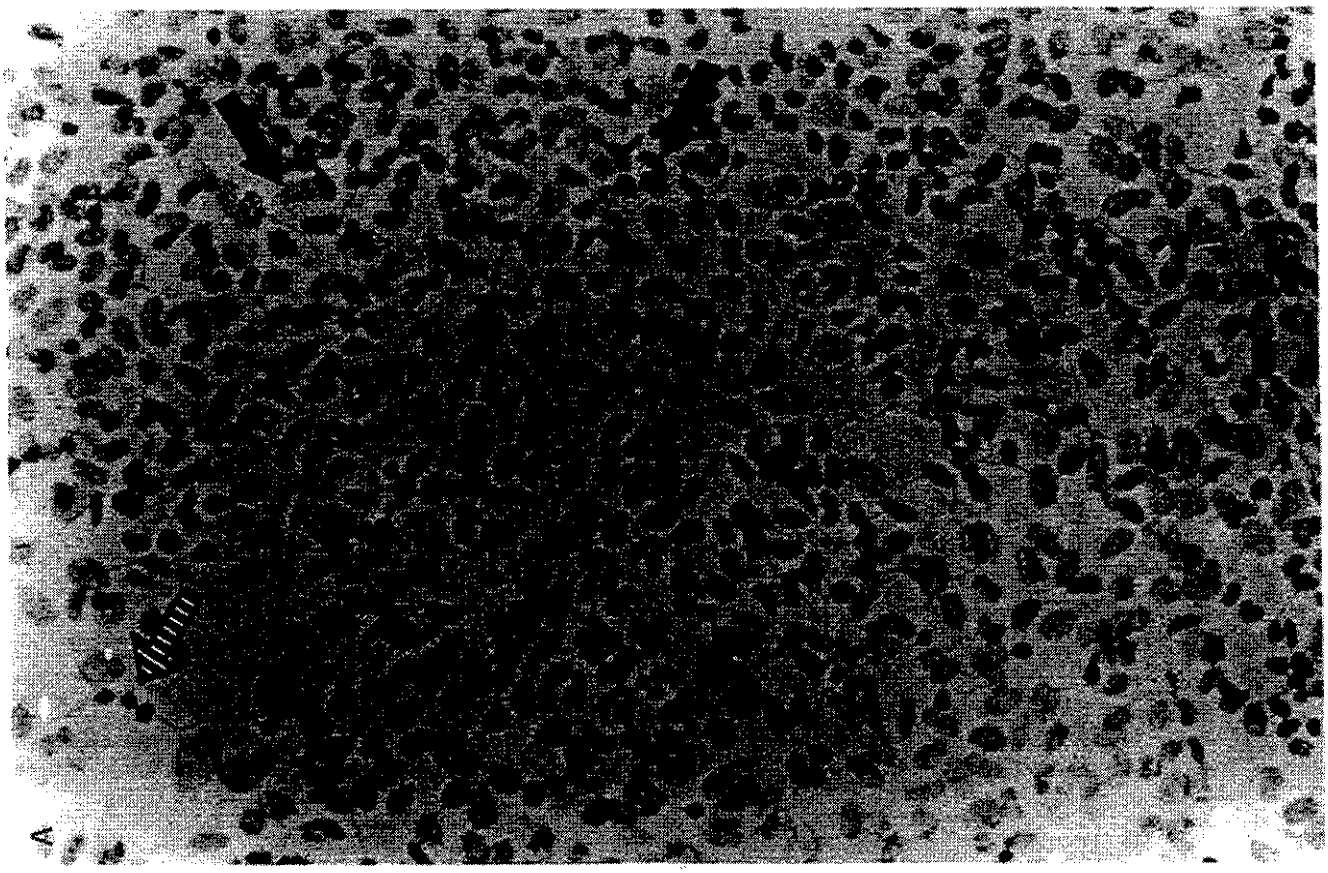
Terminal deoxynucleotide transferase (TdT) labeling for in situ analysis of apoptosis. *In situ* analysis of DNA integrity was assessed using the protocol of Tilly *et al.* (1995). Bone was fixed and decalcified (Surgipath, Richmond, IL) for 2 days, changing the solution each day. Tissue was rinsed in water for 1 hr, embedded in paraffin, sectioned, and mounted unstained onto microscope slides. Paraffin was removed by incubating for 30 min in a 60°C oven followed by a 10-min wash in xylenes. Tissue was rehydrated with graded concentrations of ethanol as follows: 100% ethanol for 10 min, 90% ethanol for 3 min, 2% H_2O_2 in 90% methanol for 1 min, 80% ethanol for 3 min, 70% ethanol for 3 min, and sterile water for 3 min. Each section was treated with proteinase K (10 μg/ml in 2 mM $CaCl_2$, 20 mM Tris–HCl, pH 7.4) and incubated for 30 min at 37°C. Slides were washed twice with sterile water. Tissue was preequilibrated with 5× TdT reaction buffer provided by the manufacturer and $CoCl_2$ (Boehringer-Mannheim, Indianapolis, IN) for 20 min. Slides were then incubated for 15 min at 37°C with the reaction mixture for 3'-end labeling. The reaction mixture consisted of TdT reaction buffer, 5 mM $CoCl_2$, 50 μM biotin-16-dUTP, 500 μM dUTP, and 25 units/0.1 ml TdT enzyme (Boehringer–Mannheim). Slides were washed three times for 10 min each in 150 mM NaCl, 100 mM Tris–HCl (pH 7.5) buffer and then blocked by incubation with 3% BSA (w/v) in buffer for 30 min at room temperature. ABC reagent (Vector Labs, Inc., Burlingame, CA) was added, and slides were incubated at room temperature for 15 min, dipped in buffer (100 mM NaCl, 100 mM Tris–HCl, pH 9.5, and 50 mM $MgCl_2$), and washed three times for 10 min each in buffer. DAB (Vector Labs) was used to detect localization of incorporated biotin-dUTP in apoptotic cells. Reactions were stopped with TE buffer and slides counterstained with hematoxylin, followed by a lithium carbonate wash. Sections were dehydrated with a graded ethanol series (70–100% ethanol; 1 min each) and allowed to air dry before coverslip mounting with Permount.

RESULTS

DMBA induces preB cell apoptosis. Culture of bone marrow cells under conditions described originally by Whit-

FIG. 5. *In situ* hybridization for AhR mRNA. Primary bone marrow cultures were fixed with 4% paraformaldehyde, dehydrated, and hybridized for 18 hr at 52°C with an AhR antisense ^{35}S-labeled riboprobe. Slides were washed and autoradiographs exposed for 6 weeks. Solid arrows indicate stromal cells expressing AhR mRNA signal. Striped arrows indicate preB cells. No signal was observed with AhR sense riboprobes (not shown). (A) Bright field. (B) Darkfield.





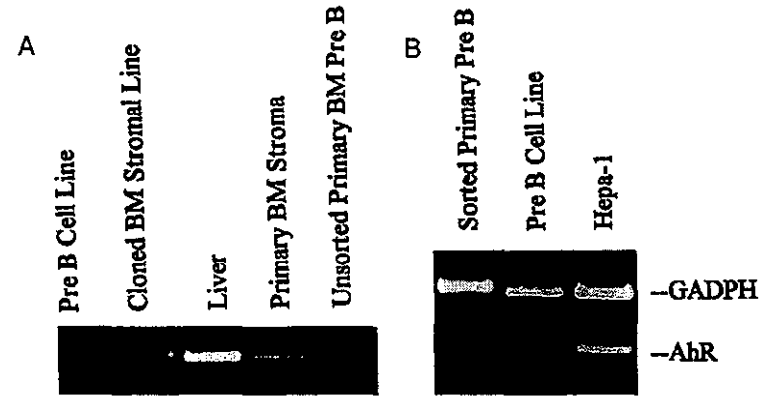


FIG. 6. RT-PCR for AhR mRNA in primary bone marrow stromal and preB cells. (A) RNA was extracted from a preB cell line (Yamaguchi *et al.*, 1996), a cloned bone marrow stromal cell line (Pietrangeli *et al.*, 1988), liver cells from C57BL/6 mice, bone marrow stromal cells from 4-week-old bone marrow cultures, or preB cells harvested from 4-week-old bone marrow cultures (90% CD45/B220$^+$). RNA was reverse transcribed and cDNA amplified with AhR- and GADPH-specific primers. Samples were normalized according to the GADPH signal. The predominant AhR band is shown. (B) RNA was extracted from fluorescence-activated cell-sorted preB cells from 4-week-old bone marrow cultures (>95% CD45/B220$^+$), a preB cell line (Yamaguchi *et al.*, 1996) and Hepa-1c1c7 hepatoma cells (Cuthill *et al.*, 1987). RNA was reverse transcribed and cDNA amplified simultaneously with AhR- and GADPH-specific primers.

lock *et al.* (1984) resulted in the outgrowth of CD45/B220$^+$, sIg$^-$ B cell populations expressing rearranged immunoglobulin heavy chain genes, i.e., preB cell populations (Fig. 1). It was noted that addition of 10^{-5} M DMBA to these cultures resulted in the disappearance of preB cells within 7 days (data not shown). Examination of preB cells from DMBA-treated cultures by light microscopy revealed cellular and nuclear condensation characteristic of apoptosis. To confirm that DMBA induces preB cell apoptosis, primary bone marrow cultures were treated with vehicle or with 10^{-5} M DMBA. PreB cells were harvested 18 and 24 hr later, and DNA was extracted for analysis of fragmentation characteristic of apoptosis (Ju *et al.*, 1995). A low but discernible level of digestion of DNA into oligonucleosomal (i.e., multiples of 200 base pairs) fragments was detected in control cultures (Fig. 2). Exposure of cultures to 10^{-5} M DMBA for 24 hr resulted in a dramatic increase in DNA fragmentation. In other experiments not shown, increased DNA fragmentation was evident within 18 hr of DMBA exposure. These results are consistent with DMBA-mediated preB cell apoptosis.

To quantitate apoptosis, cultures were treated with vehicle or DMBA. PreB cells were harvested 18 to 24 hr later and the proportion undergoing apoptosis was quantitated by DNA staining with propidium iodide and by flow cytometric analyses of cell morphology (Hardin *et al.*, 1992). Data from one experiment (from over 20 total) are presented in Fig. 3. Relatively few (8%) vehicle-treated cells exhibited a dull PI staining pattern characteristic of cells undergoing apoptosis. Similarly, few vehicle-treated cells (10%) exhibited the classic morphologic features of apoptotic cells, i.e., smaller (lower forward scatter) and more granular (increased side scatter). The percentage of apoptotic cells, as defined by both morphologic and DNA staining parameters, increased to approximately 40 and 50% following DMBA exposure for 18 and 24 hr, respectively. In contrast, bone marrow stromal cells were resistant to DMBA, demonstrating no change in PI staining 24 hr after DMBA exposure (data not shown). Collectively, the data confirm that DMBA induces preB cell apoptosis in primary bone marrow cultures.

Extensive kinetics and titration experiments indicated that significant levels of apoptosis, as assessed both by the PI staining pattern and by morphologic criteria (forward and side light-scatter parameters) were induced after 24 hr with as little as 10^{-6} M DMBA and after 48 hr with as little as 10^{-8} M DMBA (Fig. 4, $p < 0.05$). The percentage of cells undergoing apoptosis continued to rise 72 hr after exposure to 10^{-8} M DMBA.

Expression of AhR mRNA and protein in bone marrow cultures. Since the AhR has been implicated in PAH-in-

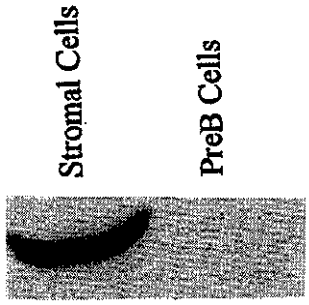
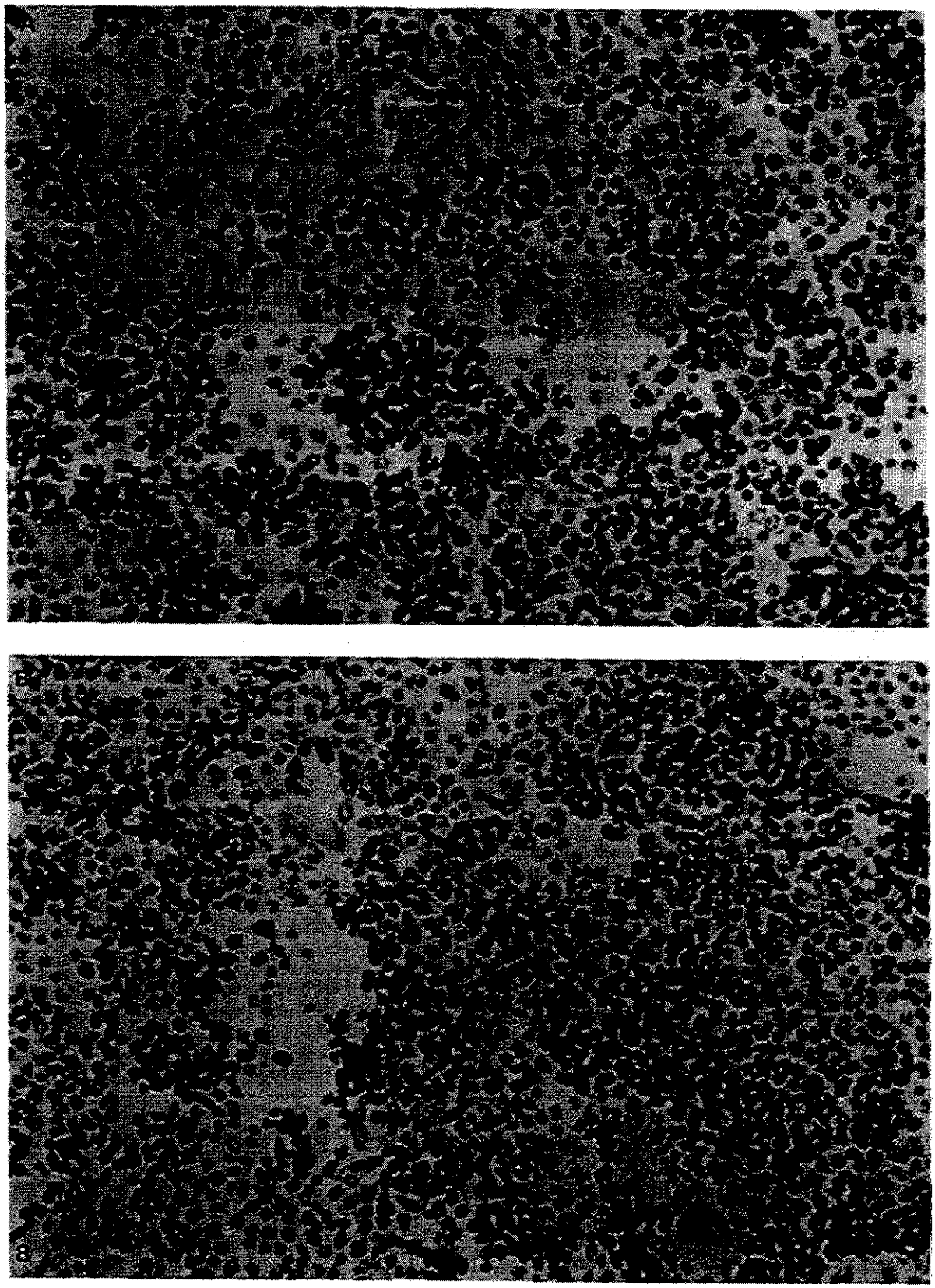
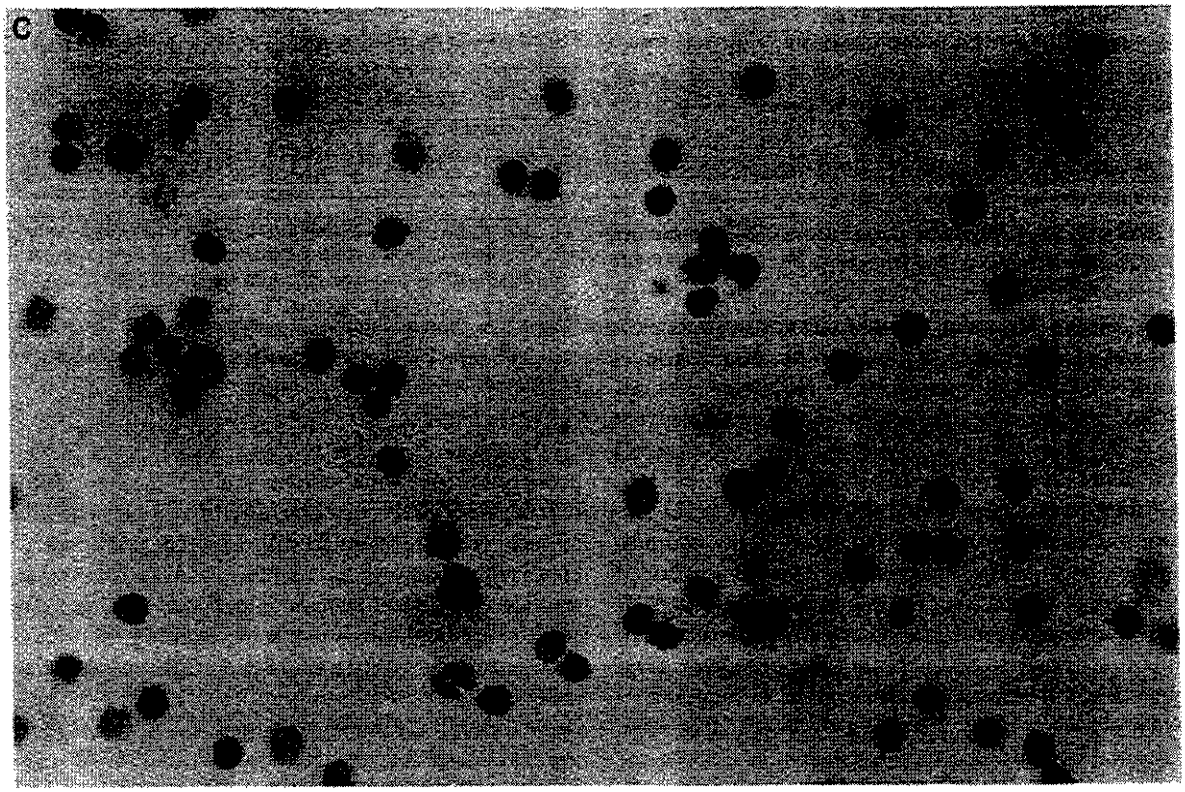


FIG. 7. Immunoblot for AhR in bone marrow stromal and preB cells. Total cellular protein from untreated stromal cells and preB cells from 4-week-old bone marrow cultures was extracted, electrophoresed, transferred to nitrocellulose filters, and immunoblotted with monoclonal (RPT-1) anti-AhR antibody. The predominant 95- to 100-kDa AhR band is shown. Data from a representative experiment are presented.

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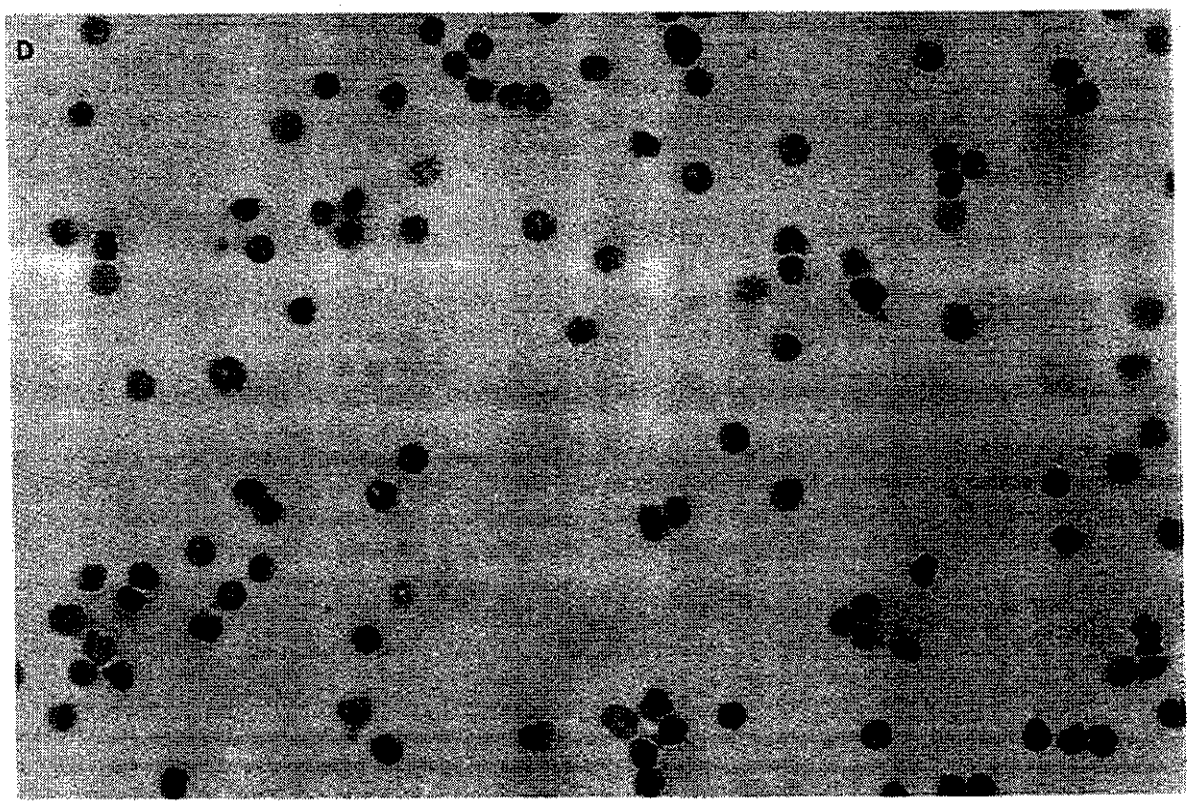
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duced immunosuppression (Hardin *et al.*, 1992; Kerkvliet *et al.*, 1990; Morris *et al.*, 1994; Silkworth *et al.*, 1984; Thurmond *et al.*, 1988; White *et al.*, 1985; Wojdani *et al.*, 1984; Harper *et al.*, 1994), and since little is known about AhR expression either in immature lymphocyte populations or in the stromal cells which in part constitute the bone marrow microenvironment, *in situ* hybridization was employed to evaluate AhR mRNA expression in cultures of bone marrow-derived preB and stromal cells. Interestingly, a strong AhR mRNA signal was detected in some bone marrow stromal cells (Figs. 5A and 5B, solid arrows) but not in preB cells (striped arrow). Great variability was observed in the level of AhR mRNA signal in stromal cells. No signal was detected when probing with an AhR sense riboprobe (not shown).

A more sensitive technique, AhR mRNA-specific RT-PCR, was then used to confirm results obtained by *in situ* hybridization. Consistent with *in situ* hybridization studies, significant levels of AhR mRNA were detected in a cloned bone marrow stromal cell line, BMS2, which supports preB cell growth (Pietrangeli *et al.*, 1988) and in primary stromal cell populations from 4-week-old bone marrow cultures (Fig. 6A). Results from *in situ* hybridization (Fig. 5) suggest that most of that signal is likely produced by a subset of bone marrow stromal cells. In contrast, no AhR mRNA was detected in a stromal cell-dependent preB cell line (Yamaguchi *et al.*, 1996) and minimal AhR mRNA was detected in unsorted primary preB cell populations harvested from bone marrow cultures (Fig. 6A).

To determine if this weak AhR mRNA signal in the preB cell population was due to contamination with AhR^+ stromal cells, preB cells from bone marrow cultures were stained for CD45/B220 expression and sorted on the basis of forward (size) and side (morphology) light-scatter parameters and CD45/B220 expression. Sorted B cell populations were >95% $CD45/B220^+$. No AhR mRNA was detected in these sorted primary preB cell populations (Fig. 6B), strongly suggesting that preB cells from bone marrow cultures do not express AhR.

Western immunoblotting for AhR protein was consistent with AhR mRNA assays in that significant levels of AhR were detected in primary stromal cells but not preB cell populations from bone marrow cultures (Fig. 7). These data suggest that if the AhR is involved in DMBA-induced apoptosis, then it is likely that the death signal is delivered indirectly through the stromal cell feeder layer.

Expression of AhR in fresh bone marrow isolates. To confirm that the failure to detect AhR mRNA or protein in preB cells from bone marrow cultures was not due to selection of AhR^- preB cells or to down-regulation of AhR during culture, AhR levels in freshly isolated, unfractionated bone marrow populations and in bone marrow B cell populations purified by fluorescence-activated cell sorting were evaluated by immunohistochemistry. Staining of unfractionated bone marrow cells with an AhR-specific polyclonal antibody revealed cytoplasmic staining, particularly in large, non-lymphoid cells (Fig. 8B vs negative control in Fig. 8A). In contrast, bone marrow B cells, purified by sorting on the basis of forward and 90° light-scatter parameters and CD45/B220 expression, were not stained with anti-AhR antibody (Fig. 8D vs negative control in Fig. 8C). In addition, no AhR mRNA was detected in sorted bone marrow B cells by RT-PCR (data not shown). These results are consistent with those obtained with long-term bone marrow cultures and further suggest that any AhR-dependent effects on this immature B cell population are dependent on AhR^+, non-B cells in the bone marrow microenvironment.

TABLE 1
α-Naphthoflavone Blocks DMBA-Induced Apoptosis[a]

Time	Vehicle	α-NF	DMBA	α-NF + DMBA
24 hr	6 ± 2	9 ± 1	23 ± 8	12 ± 3*
48 hr	8 ± 2	10 ± 1	35 ± 1	8 ± 2*

[a] Vehicle, 10^{-5} M α-naphthoflavone (α-NF), and/or 10^{-4} M DMBA were added to bone marrow cultures 3 weeks after culture initiation. PreB cells were harvested 24 or 48 hr later and the percentage of cells undergoing apoptosis was quantitated by the P.I., flow cytometry method. Data pooled from four experiments are presented as percentages apoptosis ± standard error. An asterisk indicates a significant decrease in percentage apoptosis, $p < 0.05$ (*t* test).

α-Naphthoflavone blocks DMBA-induced preB cell apoptosis in bone marrow cultures. If the AhR and/or AhR-regulated P450 enzymes are critical to generation of a death signal, then it would be predicted that α-naphthoflavone (α-NF), a competitive inhibitor of the AhR (Gasiewicz and Rucci, 1991; Blank *et al.*, 1987; Merchant *et al.*, 1993) and P450IA1 inhibitor (Gurtoo *et al.*, 1979), would block DMBA-induced apoptosis. As shown in Table 1, addition of 10^{-5} M α-NF had no effect on preB cell viability. In this series of four experiments, 10^{-4} M DMBA induced 23 and 35% of the preB cells to undergo apoptosis within 24 and 48 hr, respectively. However, addition of a log less α-NF to DMBA-treated cultures completely rescued preB cells from apoptosis ($p < 0.05$). This result is consistent with a

FIG. 8. Immunohistochemical analysis of AhR in fresh bone marrow cells. Bone marrow cells were expunged from the femurs of C57BL/6 mice and stained for CD45/B220 expression. Cells were sorted on the basis of forward and 90° light-scatter parameters (i.e., lymphocyte gated) and CD45/B220 fluorescence. Unsorted and sorted populations were cytospun onto glass slides, fixed, and stained for AhR protein expression. (A) Unsorted cells, immunoglobulin control; (B) unsorted cells, anti-AhR antibody; (C) CD45/B220 sorted cells, immunoglobulin control; (D) CD45/B220 sorted cells, anti-AhR antibody.

role for the AhR in preB cell death. It is also possible that the inhibitory effect of α-NF reflects a contribution of P450 enzyme activity to apoptosis. Since the AhR influences P450 activity (Cuthill *et al.*, 1987; Dolwick *et al.*, 1993; Ema *et al.*, 1992), a role for P450 would still be consistent with the ability of the AhR to influence DMBA-induced apoptosis in immature B lymphocytes.

PAH induce bone marrow cell apoptosis in vivo. To begin to extend studies from this model system of B lymphopoiesis to the bone marrow environment *in vivo*, mice were injected ip with vehicle, B[*a*]P, or DMBA. Eighteen and 48 hr later, mice were sacrificed, and femurs were removed and analyzed for apoptotic cells using an *in situ* DNA labeling assay. In this assay, apoptotic cells can be identified both by morphologic criteria (small cells with nuclear condensation and/or nuclear apoptotic bodies) and by TdT-catalyzed biotinylated nucleotide incorporation (visualized as a dark blue–brown stain). Bone marrow from vehicle-treated mice contained few apoptotic cells 18 hr (Fig. 9A) or 48 hr (Fig. 9D) after vehicle injection. In contrast, bone marrow from B[*a*]P- or DMBA-treated mice exhibited significant numbers of apoptotic cells as early as 18 hr after treatment (Figs. 9B and 9C, arrowheads) and persisting at least until 48 hr (Figs. 9E and 9F). In most sections, apoptotic cells tended to occur in clusters (Figs. 9B and 9C). Occasionally, apoptotic cells could be visualized in apparent association with stromal cells (Fig. 9C, large arrowhead). In agreement with previous reports (Nebert *et al.*, 1980), significant hypocellularity was noted 48 hr after injection with B[*a*]P or DMBA (Figs. 9E and 9F vs control in Fig. 9D). These data are consistent with PAH-mediated induction of hematopoietic cell apoptosis *in vivo*.

DISCUSSION

DMBA acts as an immunosuppressant *in vivo* and alters lymphocyte function *in vitro* (Burchiel *et al.*, 1993; Burchiel *et al.*, 1992; Ladics *et al.*, 1991). In the present work we extend these studies with an investigation into the effects of DMBA on developing bone marrow lymphocytes. Having observed that DMBA inhibits growth of preB cells in long-term bone marrow cultures, we demonstrate herein that the mechanism of this immunosuppression is, at least in part, the induction of preB cell apoptosis.

Apoptosis is a manifestation of programmed cell death and is a critical process in development of the immune system. That is, upon ligation of antigen-specific receptors, immature autoreactive lymphocytes are deleted by activation of the cell death program (Ju *et al.*, 1995). Therefore, it is of significance that DMBA can activate the cell death program regardless of lymphocyte receptor specificity. Implications of this finding include a skewing of the developing lymphocyte repertoire and/or diminution of emerging lymphocyte populations. Suppression of lymphopoiesis could result in enhanced susceptibility to infectious disease, particularly if generalizable to the T cell compartment. The latter possibility is currently under investigation.

Titration experiments demonstrate that significant levels of apoptosis are induced with as little as 10^{-8} M DMBA. DMBA-induced apoptosis is presented herein primarily as increasing percentages of cells exhibiting a sub G_0/G_1 peak, although it is also visualized by DNA fragmentation at doses as low as 10^{-8} M (Mann *et al.*, 1997), a corresponding decrease in cell size as assessed by flow cytometry, and nuclear condensation as seen by light microscopy. This PAH dose is lower than those generally reported to effect immunosuppression of mature lymphocyte populations *in vitro* (Burchiel *et al.*, 1993; Thurmond *et al.*, 1988; Ladics *et al.*, 1991), although a recent report demonstrates suppression of peripheral human lymphocyte mitogenic responses with 10^{-8} M DMBA (Davila *et al.*, 1996). Indeed, doses of DMBA capable of inducing apoptosis in the present system approach doses at which 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), generally considered to be the more potent xenobiotic, affects immune cell function (Harper *et al.*, 1995; Morris *et al.*, 1994; Luster *et al.*, 1988). Similarly, doses of B[*a*]P as low as 10^{-8} M suppressed B lymphopoiesis in bone marrow cultures (Hardin *et al.*, 1992). These results may be contrasted with those obtained with a noncarcinogenic PAH, benzo[*e*]pyrene, which binds the AhR poorly and which had no effect on B cells in bone marrow cultures at doses as high as 10^{-5} M (Hardin *et al.*, 1992). Finally, since techniques for measuring apoptosis assess cell death at a single time point, our studies may overestimate the doses of DMBA required to adversely affect immature lymphocytes in long-term cultures.

The ability of relatively low DMBA doses to effect B cell apoptosis may reflect the contribution of the stromal cell microenvironment to activation of the cell death program. That is, while DMBA doses on the order of 10^{-5} M are required to induce clonal B lymphoma cells to die (Burchiel *et al.*, 1993), concentrations of DMBA three log-orders less are required to kill preB cells maintained on bone marrow stromal cells. An important role for the lymphoid microenvironment in xenobiotic-induced immunosuppression has previously been suggested (Greenlee *et al.*, 1985; King *et al.*, 1989; Kremer *et al.*, 1994). Alternatively or in addition, the low doses required for DMBA-induced preB cell apoptosis may be due to heightened sensitivity of immature cells to xenobiotics in general.

The role of the AhR in immunosuppression of mature lymphocyte responses has been controversial (Morris *et al.*, 1994; Davis and Safe, 1991; Harper *et al.*, 1994, 1995; Thurmond *et al.*, 1988). Given the weight of evidence from both *in vivo* and *in vitro* studies (Kerkvliet *et al.*, 1990; Blank *et al.*, 1987; Morris *et al.*, 1994), it seems likely that at least part of the immunosuppression induced with AhR ligands is influenced by the AhR. Previous work from our laboratory

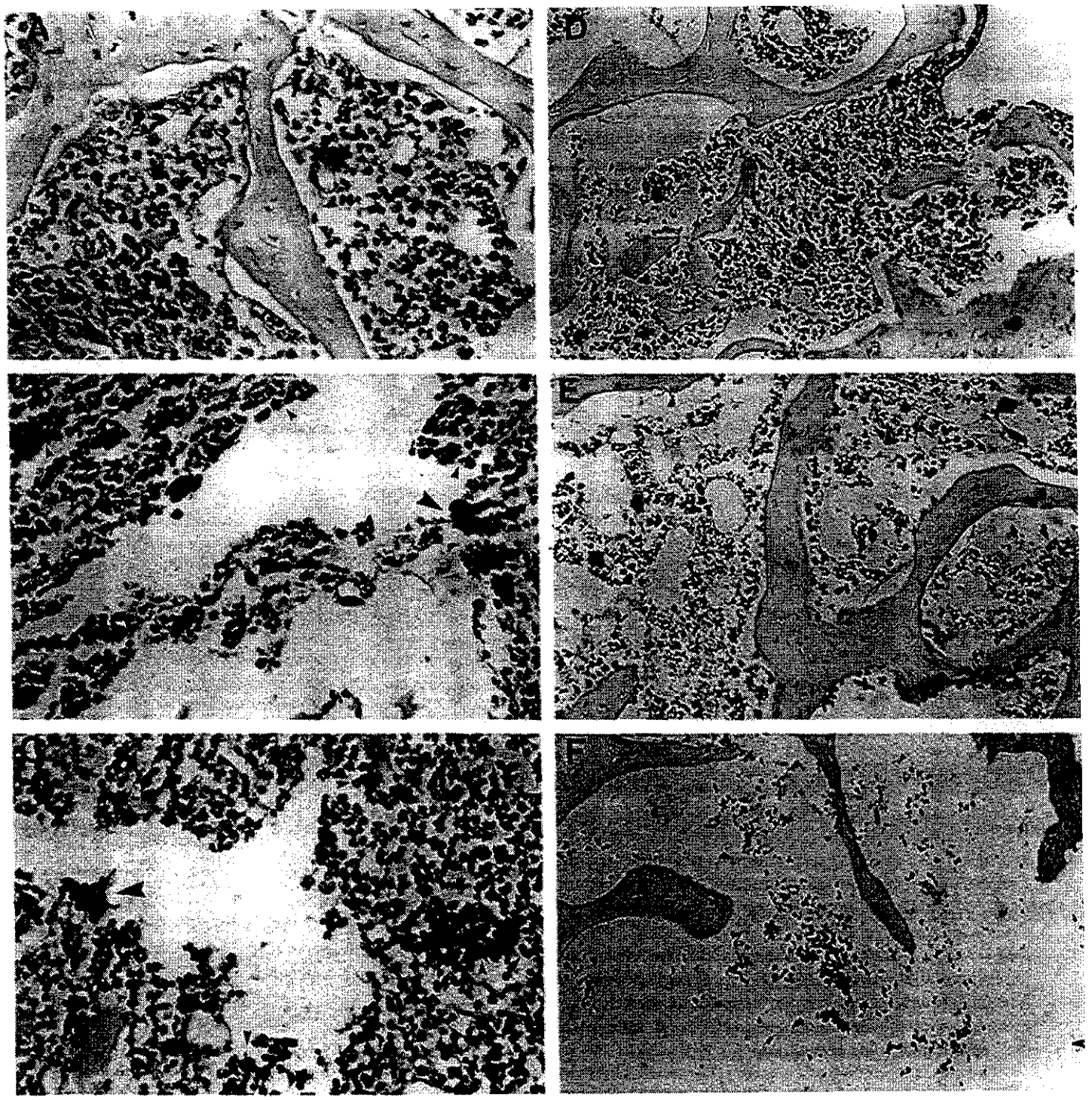

FIG. 9. *In situ* analysis of PAH-induced bone marrow cell apoptosis. C57BL/6 mice were injected ip with vehicle (0.2 ml) (A and D), 2 mg B[*a*]P (B and E), or 2 mg DMBA (C and F). Mice were sacrificed 18 (A–C; 200×) or 48 (D–F; 100×) hr later, and femurs were removed, fixed, decalcified, embedded, and analyzed for apoptotic cells *in situ* with the TdT assay. Apoptotic cells with condensed nuclei and/or DNA fragmentation (dark blue–brown TdT stain) are indicated with small arrowheads. Apoptotic cells frequently appear in clusters and sometimes in apparent association with stromal cells (large arrowheads).

has suggested a role for the AhR and/or AhR-regulated genes in B[*a*]P-mediated suppression of lymphopoiesis (Hardin *et al.*, 1992). Both B[*a*]P and DMBA induce α-NF-inhibitable AhR nuclear translocation and EROD activity (Yamaguchi *et al.*, 1997; Mann *et al.*, 1997). However, it remains to be determined in either the DMBA or B[*a*]P system if the AhR induces a death signal through second-messenger signaling (Gradin *et al.*, 1994; Enan and Matsumura, 1995), by inducing transcription of "death genes" or by indirectly influencing cell function through cytochrome P450 induction and the subsequent production of DMBA metabolites. The ability of nonlymphoid cells in the lymphoid microenvironment to metabolize PAH into compounds capable of suppressing mature lymphocyte responses is consistent with the latter possibility (Davila *et al.*, 1996; Ladics *et al.*, 1991). In any case, if the AhR and/or AhR-regulated P450 enzymes are important in PAH-induced apoptosis, then it is of particular significance that primary bone marrow stromal cells, but not preB cells from bone marrow cultures or freshly isolated bone marrow B cells, express AhR (Figs. 5–8). These results suggest a model in which low doses of AhR ligands, such as DMBA, activate AhR^+ cells in the bone marrow microenvironment which in turn deliver signals to adjacent, immature B cells to activate their cell death program. Consistent with this hypothesis are the observations that a preB cell line maintained in stromal cell supernatant but in the absence of stromal cells or preB cells maintained on AhR^- feeder cells do not die in response to either DMBA or B[*a*]P (Near *et al.*, 1997). The role of the stromal cells is further supported by preliminary data demonstrating that a >10-kDa factor capable of inducing apoptosis in preB cells is elicited from the stromal cells after a 24-hr treatment with DMBA (data not shown).

It is not yet clear if the subset of stromal cells expressing AhR, and presumably responsible for preB cell apoptosis, represents a distinct cell type or a discrete stage in stromal cell development. Consistent with the latter possibility, our laboratory has recently demonstrated that mitogenic signals increase AhR expression in fibroblasts (Vaziri *et al.*, 1997). In either case, these results suggest a scenario in which lymphocytes contacting AhR^+ stromal cell subsets undergo apoptosis following PAH exposure *in vitro*.

Since it is critical to eventually extend this model to whole animal studies, an *in situ* assay was employed to begin analysis of bone marrow cell apoptosis following *in vivo* administration of PAH. The advantage of such an assay is that it can localize individual apoptotic cells within the preserved architecture of the bone marrow environment, even when apoptotic cells are engulfed by phagocytic cells (Nakamura *et al.*, 1996). Confirmation of apoptosis using unfractionated bone marrow cell populations through gel electrophoresis or PI/FACS analyses would be problematic given the very rapid clearance of apoptotic cells *in vivo* and the prediction that apoptosis occurs primarily in a subset of cells associated with stromal cells. Nevertheless, our results clearly demonstrate that bone marrow cells, many exhibiting lymphoid morphology, undergo apoptosis shortly after injection with either B[*a*]P or DMBA. The frequent clustering of apoptotic cells and the visualization of apoptotic cells in close proximity with epithelial-like stromal cells are consistent with, but do not prove, the hypothesis that lymphocyte apoptosis in response to PAH exposure *in vivo* is a localized phenomenon dependent on contact with AhR^+ stromal cells. The challenge of experiments in progress is to perform detailed dose-response and kinetics studies *in vivo*, to definitively identify bone marrow cell subsets which undergo PAH-induced apoptosis *in vivo*, and to determine if AhR^+ stromal cells are required for apoptosis *in vivo*, as appears to be the case *in vitro*.

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