Lipopolysaccharide Activation of Murine Splenocytes and Splenic B Cells Increased the Expression of Aryl Hydrocarbon Receptor and Aryl Hydrocarbon Receptor Nuclear Translocator

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ABSTRACT

These studies characterized the profile of AhR and ARNT expression in primary splenocytes and purified splenic B cells after cellular activation with lipopolysaccharide (LPS). LPS treatment of mouse splenocytes markedly increased the magnitude of both AhR and ARNT steady state mRNA expression. AhR mRNA expression peaked at 8 hr post-LPS activation and was increased by ~5-fold compared with freshly isolated splenocytes (i.e., time 0). ARNT mRNA expression began to increase by 8 hr postactivation, peaked at approximately 48 hr and was increased by approximately 4-fold when compared with nonactivated splenocytes at time 0. Western blotting also demonstrated an increase in the relative magnitude of both the AhR and ARNT proteins in LPS activated splenocytes. Likewise, the presence of the AhR, ARNT and cytochrome P450IA1 (CYP1A1) proteins were also detected in purified primary splenic B cells, and the magnitude of protein expression was enhanced in LPS activated splenic B cells at 12 and 24 hr relative to time matched controls for each of these proteins. In summary, these findings suggest that on LPS activation the magnitude of AhR and ARNT mRNA and protein increases in both splenocytes and purified primary splenic B cells. Moreover, because the increase in the relative magnitude of CYP1A1 protein in response to LPS occurred in the absence of exogenous AhR ligand, these results suggest that B-cell activation is sufficient to induce AhR nuclear translocation and binding to dioxin-responsive elements in the promoter region of AhR-responsive genes.

TCDD is the most toxic congener of the halogenated aromatic hydrocarbon family of compounds that have been demonstrated to bind to the AhR (Poland and Knutson, 1982). Exposure to TCDD elicits a variety of biological and toxicological effects in certain rodent species including carcinogenicity (Kociba, et al., 1978), teratogenicity (Pratt, et al., 1984) and immunosuppression (Holsapple, et al., 1991). The adverse effects of TCDD in man are less well characterized, but a good correlation between TCDD exposure and chloracne has been demonstrated (Taylor, 1979). The AhR is a 95- to 110-kDa cytosolic protein that is codominantly expressed in two forms by the B6C3F1 mouse (C3H/HeN × C57BL/6) that correspond to the two AhR alleles expressed by this mouse strain (Burbach, et al., 1992; Ema, et al., 1992; Harper, et al., 1991). While no known endogenous ligands have been identified for the AhR, TCDD and related compounds are believed to act as exogenous ligands for the AhR. The putative mechanism by which these compounds elicit their broad range of effects is believed to involve translocation of the ligand receptor complex into the nucleus followed by dissociation of hsp90 proteins (Denis, et al., 1988; Ferdew, 1988; Pongratz, et al., 1992) and dimerization with ARNT (Cuthill, et al., 1987; Okey, et al., 1980; Pollenz, et al., 1994; Probst, et al., 1993). Recent work by Ma and coworkers (1996) has suggested that the ligand-receptor complex is also associated with AIP, a cytosolic protein, that dissociates from the complex before its entry into the nucleus. Following dimerization with ARNT, the ligand-receptor heterodimer can act as a transcription factor by binding to DREs located in the promoter regions of members of the AhR gene battery such as cytochrome

ABBREVIATIONS: AhR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; CYP1A1, cytochrome P450IA1; DRE, dioxin-responsive enhancer; EMSA, electrophoretic mobility shift assay; FACS, flow-activated cell sorter; FITC, fluorescein isothiocyanate; hsp90, heat shock protein 90; LPS, lipopolysaccharide; mRNA, messenger RNA; R-PE, R-phycoerythin; RT-PCR, reverse transcriptase-polymerase chain reaction; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin.

In light of the well characterized mechanism for the regulation of CYP1A1 gene expression, the immunosuppressive effects described after exposure to TCDD are also presumed to be mediated though the AhR. Despite the low levels of AhR expression elicited by leukocytes as compared with other organs that have demonstrated sensitivity to TCDD, such as the liver (Williams et al., 1996), the immune system has been demonstrated to be one of the most sensitive target organs of TCDD-mediated toxicity (reviewed in (Holsapple et al., 1991)). An important distinction between leukocytes and cells from other TCDD sensitive tissues is that leukocytes must undergo activation, proliferation and differentiation to mediate their various immune effector functions; a characteristic that may predispose these cells to modulation by TCDD.

We have previously demonstrated both the presence and functionality of the AhR and ARNT proteins in primary leukocytes (Williams et al., 1996). In addition, we have also demonstrated that leukocyte activation with a combination of a phorbol ester and calcium ionophore (phorbol ester calcium ionophore) induced AhR up-regulation, DNA binding, and increased CYP1A1 gene expression in the absence of exogenous ligand (Crawford et al., 1997). Because LPS, a well known polyclonal B cell mitogen, is ubiquitous in our environment, it is a more physiologically relevant stimulus than phorbol ester calcium ionophore. Thus the objectives of these studies were to characterize the profile of AhR and ARNT mRNA and protein expression in B6C3F1 leukocytes in the absence of exogenous AhR ligands after activation with LPS, to demonstrate the presence of both the AhR and ARNT proteins in purified primary murine splenic B cells after cellular activation with LPS and to determine if the profile of AhR and ARNT protein expression in purified primary splenic B cells correlates with the profile of AhR and ARNT protein expression in LPS activated leukocytes.

Materials and Methods

Chemicals and media. Chemicals, unless otherwise stated, were purchased from Sigma Chemical (St. Louis, MO). All enzymes used in the quantitative RT-PCR were purchased from Promega (Madison, WI), including the Taq DNA polymerase. Lipopolysaccharide was from Salmonella typhosa. All media and their components were purchased from Gibco BRL (Grand Island, NY). Complete RPMI 1640 is RPMI 1640 that is supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin sulfate, 2 mM L-glutamine, and 10% bovine calf serum (Hyclone, Logan, UT).

Animals. Virus-free B6C3F1 mice, 5 to 6 weeks of age, were purchased from Charles River Laboratories (Boston, MA). On arrival, mice were randomized, transferred to plastic cages containing a sawdust bedding (5 mice per cage) and quarantined for 1 week. Mice were provided with food (Purina Laboratory Certified Chow; Ralston Purina, St. Louis, MO) and water ad libitum. Animal holding rooms were kept at 21° to 24°C and 40% to 60% relative humidity with a 12-hr light/dark cycle.

Splenocyte protein lysate preparation. Single spleen cell suspensions without red blood cells were prepared as previously described (Williams et al., 1996). After the last wash, one cell volume of HEDGM/LAP was added and homogenized with a tight-fitting pestle. An equal volume of HEDG2OM/LAP (HEDM/LAP with 20% glycerol) was added and centrifuged at 105,000 × g for 1 hr at 4°C. The supernatant was aliquoted and stored at ~80°C before being used in Western blot analysis. Protein concentrations were determined by the Bradford protein assay (BioRad Laboratories, Hercules, CA).

Primary B cell isolation. Single spleen cell suspensions without red blood cells were prepared as noted above. After the last wash, complement mediated T cell lysis and removal of monocytes was performed as previously described (Brooks et al., 1990). Briefly, to facilitate the tagging of T cells in the preparation, the cell suspension was incubated with anti-Thy 1.2 monoclonal mouse antibody at 4°C for 45 min. Suspensions were brought up to a volume of 15 ml with complete RPMI 1640 and centrifuged at 270 × g for 10 min at 4°C. The supernatant was then discarded and the pellet was resuspended in a volume of complete RPMI 1640 such that an addition of baby rabbit complement (Pel-Freez Clinical Systems, Brown Deer, WI) the volume would be twice that used in the anti-Thy 1.2 incubation step noted above. To facilitate T cell lysis in the preparation, baby rabbit complement was added to the cell suspension using a filtered syringe and incubated for 35 min in a 37°C water bath with continuous, gentle agitation. Complete RPMI 1640 was added to the suspension to stop the action of complement such that the final volume was 30 ml. The suspension was then centrifuged as noted above, the supernatant was discarded and the pellet was resuspended in 20 ml of complete RPMI 1640. This step was repeated two more times. To facilitate the removal of monocytes, 10 ml of suspension were added to 10 ml of G-10 Sephadex (Pharmacia, Uppsala, Sweden) that had been previously washed three times with Hanks buffered salt solution (GIBCO BRL, Grand Island, NY) and incubated for 35 min in a 37°C incubator on a platform rocker that was adjusted to the slowest setting. The G-10 Sephadex-cell suspension slurry was then passed through two G-10 Sephadex packed columns. The columns were washed twice with RPMI 1640 complete media to remove any residual B cells that may have remained on the columns.

Antibody staining of purified primary B cell preparations. Cells were stained and prepared for FACS analysis as described previously (Brooks et al., 1990). Briefly, 3 × 10⁶ cells were centrifuged as noted above, the supernatant was discarded and the cells were washed twice with 1× PBS. All staining antibodies were purchased from PharMingen (San Diego, CA). Cells were incubated with the following monoclonal antibodies, either alone or in combination, in 100 µl 1% BCS-PBS azide for 20 min on ice: R-PE-conjugated anti-mouse CD45R/B220, R-PE-conjugated rat IgG2ak isotype standard, FITC-conjugated anti-mouse CD3 or FITC-conjugated rat IgG2ak isotype standard. Cells were then washed twice in 1% BCS-PBS azide. If a second antibody stain was required, cells were stained with the second antibody using the procedure described above. After the second wash of 1% BCS-PBS cells were washed twice in 1% PBS azide without BCS. Cells were then resuspended in 1 ml of 2% formaldehyde in PBS and transferred into Fisher tubes for FACS analysis using a Becton Dickinson Vantage flow cytometer. Cells were analyzed within 24 hr of staining. Data were analyzed using PC Lysis version 1.1.

Western blot analysis. Western blot analysis was performed on whole cell lysates prepared from either splenocytes or purified primary murine splenic B cells. Briefly, cell lysates were prepared in HEDGM buffer, resolved by denaturing SDS-PAGE with 7.5% polyacrylamide (National Diagnostics, Manville, NJ) and transferred to nitrocellulose as previously described (Crawford et al., 1997). Immunoblot analysis and immunochemical staining were performed as previously described (Crawford et al., 1997). Primary antibodies to the AhR (Biomol Research Laboratories, Plymouth Meeting, PA), ARNT, as previously described by Pollenz et al. (1994), and CYP1A1 (Oxford Biomedical Research, Oxford, MI) were diluted to 1 µg/ml in antibody dilution buffer.

Quantitative RT-PCR. Quantitative RT-PCR was performed as described previously (Williams et al., 1996) with several modifications. Briefly, total RNA from each sample was isolated using Tri-Reagent (Molecular Research Center, Cincinnati, OH). Total RNA (100 ng) and internal standard (recombinant RNA) were reverse
transcribed simultaneously in the same reaction tube. The AhR PCR reaction consisted of PCR reaction buffer, 4 mM MgCl₂ and 2.5 units of Taq DNA polymerase (Promega). Samples were cycled 35 times; each cycle consisted of 94°C for 15 sec, 59°C for 30 sec and 72°C for 45 sec. PCR products were visualized by ethidium bromide staining and quantified by assessing the absorbance for both of the DNA bands using a Gel Doc 1000 video imaging system (BioRad, Hercules, CA). The number of transcripts were calculated from a standard curve generated by using the density ratio between the gene of interest and the different internal standard concentrations used.

Primer sequences for the AhR are forward primer, TCATGGAGAGGTCGCTCCAGG, reverse primer, GTCTTAATCATGCGGATGTGG; primer sequences for the ARNT are: forward primer, TCCGATCCGATCTAACGACC, reverse primer, TGGTCTGATCTGCACTTGC.

Quantitative RT-PCR analysis of AhR expression in mouse splenocytes. Results of dose-response studies suggested that 500 ng/ml LPS resulted in both adequate cellular activation and excellent cell viability (data not shown). Therefore, unless otherwise stated in this and subsequent sections, cells were activated with 500 ng/ml LPS. The magnitude of AhR mRNA transcript expression in primary leukocytes began to increase relative to time matched resting control cells as early as 1 hr after LPS treatment (fig. 1). The magnitude of transcript expression continued to increase and peaked at 8 hr post-LPS activation. The relative magnitude of transcript expression began to decrease at 12 hr and approached background levels by 24 hr postactivation. AhR mRNA expression was increased by ~5-fold in LPS-activated splenocytes at 8 hr compared with freshly isolated, resting splenocytes (i.e., time 0). Isolated splenocytes not activated with LPS exhibited a relatively constant basal level of AhR mRNA expression during the first 8 hr of culture. The viability of the nonactivated cells remained above 85% for the first 8 hr of culture but by 12 hr the viability of the cells had dropped to 80%, and by 24 hr of culture without cellular activation the viability was less than 70%. Therefore, time matched nonactivated control groups were only included for time points of 24 hr or less. Antibodies to the AhR revealed both of the characteristic AhR isoforms that are ~95 and ~104 kDa. These two sizes correspond to the codominantly expressed AhRb-1 (C57BL/6) and AhRb-2 (C3H/HeN) alleles in B6C3F1 mice (Burbach et al., 1992; Ema et al., 1992; Harper et al., 1991). Background AhR protein expression began to decline by 12 hr in nonactivated splenocytes. After cellular activation, splenocytes demonstrated an increase in the relative magnitude of AhR protein expression beginning at 2 hr postactivation relative to time matched resting controls. The relative magnitude of AhR protein expression continued to increase though 8 hr, peaked at 12 hr postactivation and declined slightly at 24 hr postactivation but remained markedly elevated relative to time matched resting controls from 24 hr throughout the duration of the time course.

Preliminary studies demonstrated the presence of the AhR protein in nonactivated purified primary splenic B cells (fig. 3A) (Note: The purity of the B cells was determined to be 92% by FACS analysis; data not shown.) Abbreviated time courses were then performed after cellular activation that demonstrate a profile of AhR protein expression that was similar to that seen in activated splenocytes (fig. 4).

Western analysis of AhR protein expression in mouse splenocytes and purified primary murine splenic B cells. As noted above, we have previously demonstrated the presence and functionality of the AhR and ARNT proteins in splenocytes by EMSA using nuclear proteins isolated from TCDD-treated splenocytes (Williams et al., 1996). Moreover, our laboratory has also demonstrated that leukocyte activation induced AhR up-regulation, DNA binding and increased CYP1A1 expression after cellular activation with phorbol ester and calcium ionophore (Crawford et al., 1997). In the present experiments, the profile of AhR protein expression in mouse splenocytes was characterized over a time course spanning 72 hr in the presence and absence of cellular activation as indicated in figure 2. Again, as noted above, the viability of the nonactivated cells remained above 85% for the first 8 hr of culture but by 12 hr the viability of the cells had dropped to 80%, and by 24 hr of culture without cellular activation the viability was less than 70%. Therefore, time matched nonactivated control groups were only included for time points of 24 hr or less. Antibodies to the AhR revealed both of the characteristic AhR isoforms that are ~95 and ~104 kDa. These two sizes correspond to the codominantly expressed AhRb-1 (C57BL/6) and AhRb-2 (C3H/HeN) alleles in B6C3F1 mice (Burbach et al., 1992; Ema et al., 1992; Harper et al., 1991). Background AhR protein expression began to decline by 12 hr in nonactivated splenocytes. After cellular activation, splenocytes demonstrated an increase in the relative magnitude of AhR protein expression beginning at 2 hr postactivation relative to time matched resting controls. The relative magnitude of AhR protein expression continued to increase though 8 hr, peaked at 12 hr postactivation and declined slightly at 24 hr postactivation but remained markedly elevated relative to time matched resting controls from 24 hr throughout the duration of the time course.

Quantitative RT-PCR analysis of ARNT mRNA expression in mouse splenocytes. The magnitude of ARNT
Methods and treated with 500 ng/ml LPS for various times. Then, 100 μg of total cellular protein was loaded in each lane and resolved on a 7.5% SDS-PAGE gel, transferred to nitrocellulose and incubated with either 1.5 μg/ml of rabbit polyclonal antibody for the AhR protein (Biomol) or 1.0 μg/ml of a rabbit polyclonal anti-ARNT 20–9B antibody for the ARNT protein. Antibody staining was detected by staining the blot with an anti-rabbit horseradish peroxidase-linked immunoglobulin. The relative intensity for antibody binding was detected by staining the blot with an anti-rabbit horseradish peroxidase-linked immunoglobulin. This figure is representative of three separate experiments.

Fig. 3. Expression of AhR (A) and ARNT (B) proteins in nonactivated purified primary splenic B cells as assessed by Western blotting. Primary B cells (5 × 10⁶ cells/ml) were purified as described in Materials and Methods. Then, 100 μg of total cellular protein was loaded in each lane and resolved on a 7.5% SDS-PAGE gel, transferred to nitrocellulose and incubated with either 1.5 μg/ml of rabbit polyclonal antibody for the AhR protein (Biomol) or 1.0 μg/ml of a rabbit polyclonal anti-ARNT 20–9B antibody for the ARNT protein. Antibody staining was detected by staining the blot with an anti-rabbit horseradish peroxidase-linked immunoglobulin. This figure is representative of four separate experiments.

Fig. 4. Expression of AhR protein in nonactivated vs. LPS-activated purified primary splenic B cells as assessed by Western blotting. Primary B cells (5 × 10⁶ cells/ml) were purified as described in Materials and Methods and treated with 500 ng/ml LPS for various times. Then, 100 μg of total cellular protein was loaded in each lane and resolved on a 7.5% SDS-PAGE gel, transferred to nitrocellulose and incubated with 1.5 μg/ml of a rabbit polyclonal antibody for the AhR protein (Biomol). Antibody binding was detected by staining the blot with an anti-rabbit horseradish peroxidase-linked immunoglobulin. The relative intensity for the 0 time treatment group was arbitrarily assigned a value of 1.00 to which all other treatment groups are compared. This figure is representative of four separate experiments.

Fig. 5. Time course of ARNT mRNA expression in nonactivated vs. activated leukocytes. Spleenocytes (5 × 10⁶ cells/ml) were treated with 500 ng/ml LPS for various times and mRNA was isolated as detailed in Materials and Methods. Quantitative RT-PCR for ARNT was performed on RNA from each time point. Viabilities are detailed in the Results section. The data are expressed as the mean ± S.E.M. of samples from two independent experiments.

Discussion

In addition to findings reported by our laboratory (Holzapple et al., 1986), several recent lines of evidence have suggested a role for the AhR in cell cycle progression and/or differentiation that demonstrate the differential expression of AhR in mouse leukocytes after activation with phorbol ester-calcium ionophore (Crawford et al., 1997; Hayashi et al., 1995; Ma and Whitlock, 1996; Weiss et al., 1996). In previous studies (Crawford et al., 1997), we showed that AhR steady state mRNA and protein expression was rapidly increased by phorbol ester-calcium ionophore treatment of leukocytes. In the present studies, we show that leukocyte activation by a more physiologically relevant cellular activator, LPS, produced a similar profile of differential AhR expression. Moreover, examination of purified splenic B cells demonstrated that this cell type does in fact express AhR, an observation that is consistent with previous reports from our laboratory that identified the B cell as a sensitive target for TCDD. It is also important to note that LPS induced an up-regulation of the AhR in purified splenic B cells in a manner similar to that observed in the splenocyte preparations. These findings further demonstrate that the level of AhR expression markedly fluctuates in leukocytes and suggests that the extent to that AhR is expressed may be the same as those described above for AhR mRNA expression.

Western analysis of ARNT protein expression in mouse splenocytes and purified primary murine splenic B cells. In primary splenocytes, antibodies to ARNT identified an ~87-kDa protein whose temporal profile of expression was similar to AhR. Steady state levels of ARNT protein were present in resting cells through the 24-hr time point. After cellular activation the relative magnitude of ARNT protein expression began to increase at 12 hr and remained elevated relative to time matched resting controls throughout the duration of the time course (fig. 6). Viabilities are the same as those described above for the AhR.

Preliminary studies also identified the presence of ARNT protein in nonactivated purified primary murine splenic B cells (fig. 3B). Abbreviated time courses demonstrated a profile of ARNT protein expression that was similar to that seen in activated splenocytes (figs. 6 and 7). Finally, anti-CYP1A1 antibody also identified a 53.2-kDa protein that increased at 24 hr after cellular activation with LPS (fig. 8).

Fig. 6. Time course of ARNT protein expression in nonactivated vs. LPS-activated leukocytes as assessed by Western blotting. Spleenocytes (5 × 10⁶ cells/ml) were treated with 500 ng/ml LPS for various times. Then, 100 μg of total cellular protein was loaded in each lane and resolved on a 7.5% SDS-PAGE gel, transferred to nitrocellulose and incubated with 1.0 μg/ml of anti-ARNT 20–9B, a rabbit polyclonal antibody for the ARNT protein. Antibody binding was detected by staining the blot with an anti-rabbit horseradish peroxidase-linked immunoglobulin. The relative intensity for the 0 time treatment group was arbitrarily assigned a value of 1.00 to all other treatment groups are compared. This figure is representative of four separate experiments.
of total cellular protein was loaded in each lane and resolved on a 7.5% SDS-PAGE gel, transferred to nitrocellulose and incubated with 1.0 μg/ml of a rabbit polyclonal antibody for the ARNT protein (generously supplied by Dr. Richard Pollenz). Antibody binding was detected by staining the blot with an anti-rabbit horseradish peroxidase-linked immunoglobulin. The relative intensity for the 12-hr non-LPS-activated group was arbitrarily assigned a value of 1.00 to which all other treatment groups are compared. This figure is representative of three separate experiments.

![Fig. 7. Expression of ARNT protein in non-activated purified primary splenic B cells as assessed by Western blotting. Primary B cells (5 × 10⁶ cells/ml) were purified as described in Materials and Methods and treated with 500 ng/ml LPS for various times. Then, 100 μg of total cellular protein was loaded in each lane and resolved on a 7.5% SDS-PAGE gel, transferred to nitrocellulose and incubated with 1.0 μg/ml of a rabbit polyclonal antibody for the ARNT protein (Oxford Research). Antibody binding was detected by staining the blot with an anti-rabbit horseradish peroxidase-linked immunoglobulin. The relative intensity for the 0 time treatment group was 1.00.](Image)

![Fig. 8. Expression of CYP1A1 protein in LPS-activated purified primary splenic B cells as assessed by Western blotting. Primary B cells (5 × 10⁶ cells/ml) were purified as described in Materials and Methods and treated with 500 ng/ml LPS. Then, 100 μg of total cellular protein was loaded in each lane and resolved on a 7.5% SDS-PAGE gel, transferred to nitrocellulose and incubated with 1.0 μg/ml of mouse monoclonal antibody for the CYP1A1 protein (Oxford Research). Antibody binding was detected by staining the blot with an anti-rabbit horseradish peroxidase-linked immunoglobulin. The relative intensity for the 0 time treatment group was 1.00. This figure is representative of three separate experiments.](Image)

AhR and ARNT Expression in Activated Spleen Cells

Equally interesting was the observation that concomitant with AhR up-regulation after activation by LPS, splenic B cells exhibited induction of CYP1A1 in the absence of exogenous receptor ligand. As has been widely characterized, CYP1A1 induction is mediated through the binding of parent dichotomy between the exquisite sensitivity of the immune system to inhibition by TCDD despite past findings that suggested that immunocompetent cells express surprisingly low amounts of AhR. It is notable that only nonactivated leukocytes were assayed in those studies (Williams et al., 1996).

It is also notable that the present studies differ from a recent report in that Lawrence and coworkers were unable to detect AhR in splenic B cells 72 hr after cellular activation with LPS (1996). Their inability to detect AhR in the splenic B cells may be partly due to the fact that the authors did not characterize the time course of protein expression and only assayed for AhR at 72 hr. Our studies showed a decrease in AhR expression at 24 hr after LPS activation when compared with the magnitude of AhR expression at 12 hr post-LPS treatment. Another important factor that may explain the difference between our results and those reported by Lawrence and coworkers (1996) pertains to the concentration of LPS utilized for cellular activation. Lawrence and coworkers report activating B cells with 100 μg/ml LPS but failed to discuss either how this concentration was selected or the effect it produced on cell viability. We believe this is a critical issue based on our own preliminary dose-response studies with LPS that revealed considerable lot to lot variability. However, more importantly, our preliminary dose-response studies clearly demonstrated that treatment of leukocytes for 72 hr with LPS at concentrations greater than 30 μg/ml resulted in a significant decrease in cell viability compared with cells cultured at lower LPS concentrations (data not shown). Based on the excellent cell viability observed by cells cultured at 500 ng/ml LPS, this concentration was ultimately selected for the present experiments.

The kinetics and magnitude of ARNT mRNA expression were found to differ from those observed for AhR expression. Peak ARNT mRNA expression was observed at 48 hr as compared with AhR that peaked at 12 hr. Moreover, our studies support earlier findings that demonstrated that rest-
AhR/ARNT complexes to dioxin response elements in the promoter enhancer region of the CYP1A1 gene. It is unclear why AhR undergoes nuclear translocation during leukocyte activation in the absence of PCDD's but it is tempting to speculate that perhaps this may be mediated through endogeous ligand binding.

Western blotting for CYP1A1 protein in the purified splenic B cell population demonstrated that the relative magnitude of CYP1A1 protein expression was increased at both 12 and 24 hr postcellular activation as compared with time matched vehicle controls and correlates with the time of peak AhR expression. It is notable that a similar induction of CYP1A1 has been demonstrated in mitogen activated human peripheral blood leukocytes and phorbol estercalcium ionophore activated mouse spleenocytes (Crawford et al., 1997; Vanden Heuvel et al., 1994). In the case of the phorbol estercalcium ionophore activated cells, the authors demonstrated that concomitant with the induction of CYP1A1 there was an increase of AhR/ARNT nuclear translocation and DRE binding that occurred in the absence of exogenous ligand (i.e., TCDD or structurally related AhR ligands).

In summary, the results from the present studies indicate that activation of either spleenocytes or purified splenic B cells with LPS results in an up-regulation of AhR. Furthermore, these studies demonstrate that the differential expression of AhR after phorbol estercalcium ionophore treatment is not an artifact of pharmacological activation of leukocytes because the polyclonal B cell activator, LPS, produced a similar effect. Likewise, a number of different leukocyte activators in a variety of different cell preparations have been found to induce up-regulation of the AhR and/or CYP1A1 as demonstrated by: phorbol estercalcium ionophore activation of mouse spleenocytes and human monocytes (Crawford et al., 1997; Hayashi et al., 1995); LPS activation of mouse spleenocytes and purified splenic B cells; and pokeweed mitogen-Con A activation of human peripheral blood monocytes (Vanden Heuvel et al., 1993). These findings strongly suggest that AhR up-regulation is very likely a general consequence of leukocyte activation. Moreover, the results are consistent with the time course of TCDD sensitivity after stimulation with LPS (Holsapple et al., 1986) and with the observation that resting cells are refractory to TCDD, whereas activated cells are sensitive (Morris et al., 1993). Further studies are under way to determine if the AhR plays a role in the normal progression of the B cell through its activational cell cycle.

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References
Vanden Heuvel J, Tyson F and Bell D (1993); LPS activation of mouse spleenocytes from C57BL/6 x C3H1F1 and DBA/2 mice. Immunopharmacology 12:175–186.
Pennello RS, Sattler CA and Poland PA (1994) The aryl hydrocarbon receptor and aryl hydrocarbon receptor nuclear translocator protein show distinct subcellular localizations in Hepa 1c1c7 cells by immunofluorescence microscopy. Mol Pharmacol 51:275–284.
Pollenz RS, Sattler CA and Poland PA (1994) The aryl hydrocarbon receptor and aryl hydrocarbon receptor nuclear translocator protein show distinct subcellular localizations in Hepa 1c1c7 cells by immunofluorescence microscopy. Mol Pharmacol 45:428–438.