Effects of postnatal exposure to benzo[a]pyrene on the immunity of immature rats

J. Matiasovic, L. Leva, J. Maskova, V. Kummer, M. Faldyna

Veterinary Research Institute, Brno, Czech Republic

ABSTRACT: To determine the effect of benzo[a]pyrene (B[a]P) on the developing immune system of immature rats, newborn animals received different concentrations (0.1, 1.0 and 10.0 mg/kg/day) of B[a]P in the first 14 days after birth. These rats were euthanised on day 23 of the experiment and it was found that the decreasing numbers of erythrocytes were in direct correlation to the dose of B[a]P. In subject animals, the overall counts of T cells in the spleen and the mitogenic activity increased. A decrease in the production of both IFN-γ and IL-4 (measured at the mRNA level) as well as the decreasing IFN-γ/IL-4 ratio in rat splenocytes were dependent on the dose of B[a]P. From this we may deduce that exposure to B[a]P in immature animals may modulate the immune response to infection and/or vaccination.

Keywords: polycyclic aromatic hydrocarbons; immunity; IFN-γ; IL-4; cytokines

Polycyclic aromatic hydrocarbons (PAHs) are mostly anthropogenic in origin and are released both as a result of incomplete combustion in coal, oil, gasoline and wood and in consequence of some other pyrolytic processes (Motelay-Massei et al., 2007). Benzo[a]pyrene (B[a]P) is widely used as a prototypical PAH which exhibits significant genotoxicity and negative effects on different components of the immune system. The impact of B[a]P on the immune system varies from reduced humoral immunity to increased T cell mitogenic activity (Davila et al., 1996; Burchiel and Luster, 2001; Burchiel et al., 2004). Unfortunately, there is some evidence that a comparison of the effects of pure B[a]P and diesel exhaust (DE) does not indicate any correlation between them (Burchiel et al., 2004), probably due to the complex composition of DE and a low content of B[a]P (Harper et al., 1996).

In earlier studies, it was the toxic effects of B[a]P that were mainly described and decreases in humoral and cell-mediated immunity were documented (Ward et al., 1984; Dean et al., 1986). The differences in the sensitivity of humans and rodents to varying doses of B[a]P were also described and the observation that humans were 10–100 times more sensitive to toxic effects of B[a]P than rodents was made (Davila et al., 1996). Depending on their doses, different fractions of the reconstituted PAH mixture show different effect on the humoral and cell-mediated immunity in mouse splenocytes (Harper et al., 1996). Interestingly, in more recent studies, signs of a possible adjuvant effect of PAHs or DE were shown. For example, increasing *in vitro* mitogenic activity of mouse spleen T cells in the presence of 1 μM B[a]P was documented (Burchiel et al., 2004). Also Fujimaki et al. (2001) found an increasing production of IFN-γ but not IL-4 and IL-5 by splenocytes in mice exposed to DE. In contrast, the work of Diaz-Sanchez et al. (1997) showed a decreased expression of mRNA for Th1-type cytokines (IFN-γ and IL-2) but an elevated expression for other cytokines (IL-4, 5, 6, 10, and 13) in human cells isolated from nasal lavages after stimulation by ragweed allergens and exposure to DE particles. The mechanism of this effect is not clear, but seems to be complex due to possible interaction between PAH metabolites and different lymphocyte signalling pathways such as the aryl hydrocarbon

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receptor (AhR), estrogen receptors (ERs), elevated Ca^{2+} and reactive oxygen species (ROS), the electrophilic response element and direct DNA damage (Burchiel and Luster, 2001).

Since all the data published so far had been obtained using adult subjects, the aim of the study presented here was to determine the effect of different doses of B[a]P on the developing immune system of immature rats. Newborn animals are known to have depressed lymphocyte activity and their lymphocyte subset distribution undergoes a number of changes (Adkins, 1999). In addition to this, cytokine production is impaired (Barrios et al., 1996; Adkins and Du, 1998; Rowe et al., 2000). The experiment was therefore focused on the elucidation of the ability of B[a]P to shift the Th1/Th2 balance during the response to phorbol-myristate-acetate (PMA) stimulation of isolated splenocytes. Changes in hematological parameters, lymphocyte subset distribution in the spleen and the ability to respond to a mitogen *in vitro* stimulation were also tested.

MATERIAL AND METHODS

Animals and the treatment

Pregnant Wistar rats (6–7 months old, 350 to 390 g body weight) obtained from BioTest s.r.o. (Konarovice, Czech Republic) were individually kept in temperatures between 20–24°C with a constant 12 h light/dark cycle. Animals were fed *ad libitum* with a pelleted diet (M3, Reg. No. 10250-1, VKS Jirikovice, Czech Republic) and received tap water. Within 24 h of birth, the pups were redistributed at 10 pups (both females and males) per dam, if necessary. On the whole 10 female pups from two litters were included in each experimental group. Within the first 14 days of life, the pups were injected daily with 0.1, 1.0 and 10.0 mg/kg of benzo[a]pyrene (B[a]P; CAS No. 50-32-8, Sigma-Aldrich Chemie Gmbh, Germany). 160 mg of B[a]P was suspended in 1 ml of 95% ethanol and then diluted in 19 ml of corn oil (CAS No. 8001-30-7, Fluka Chemie AG, Switzerland). The required concentration of B[a]P in application doses was adjusted by further dilution in corn oil. A total volume of 25 µl of this solution was injected subcutaneously. The B[a]P solution was vigorously shaken before each dilution and/or application. The control group was treated with corn oil only. The rats were weaned when 20 days old and euthanised by decapitation under ether anaesthesia on day 23. Before decapitation all animals were weighed. The animals were treated in accordance with the regulations of the Branch Commission for Animal Welfare of the Ministry of Agriculture of the Czech Republic. Samples of heparinised blood were taken for haematological analyses. Splenocytes were isolated by squeezing the spleen through a sterile silon (nylon) monofilament fabric into RPMI 1640 medium (Sigma, Germany) supplemented with 5% bovine foetal serum, 100 000 IU/l of penicillin (Biotika, Slovak Republic), and 0.2 g/l of streptomycin (Sigma, Germany). The remaining erythrocytes were removed by treating the isolated cells with 0.87% NH_4Cl. Purified splenocytes were resuspended in RPMI 1640 medium (supplemented with 5% bovine foetal serum, penicillin and streptomycin) until used.

Haematological analysis

The total erythrocyte and leukocyte counts in blood samples were determined using the Digicell 500 cell counter (Contraves AG, Switzerland). Differential leukocyte counts were calculated from blood smears stained according to the May-Grunwald and Giemsa-Romanowski methods.

Determination of lymphocyte subsets

Lymphocyte subsets in the spleen were analysed by flow cytometry and commercially available directly fluorochrome-conjugated monoclonal antibodies. The monoclonal antibodies (mAbs) used in this study were as follows: mouse anti-rat CD4 (clone W3/25, 10 µl per sample), CD8 (clone OX8, 10 µl per sample), γδ TCR (clone V65, 10 µl per sample) and IgM (clone MARM-4, 1 µl per sample). All monoclonal antibodies originated from Serotec (UK).

For flow cytometry, cells were resuspended in a washing and staining buffer (WSB – PBS with 0.2% gelatine from cold water fish skin, 0.1% sodium azide and 0.05mM EDTA, all reagents were from Sigma) to the density of $6–8 \times 10^6$/ml. Gelatine from cold water fish skin was used to increase mAb binding specificity as well as cell viability in flow cytometry analysis. One hundred microliters of the cell suspension was mixed with appropriate amounts of mAbs and incubated at 4°C for 20 min. Thereafter 3 ml of WSB were added, the samples

were centrifuged and the supernatants were discarded. Cells were resuspended in WSB and supplemented with propidium iodide. Propidium iodide was used to stain DNA in dead and damaged cells to exclude them from analyses.

Data were acquired on a standard FACSCalibur™ flow cytometer (Becton Dickinson, Mountain View, CA, USA) operated by CELLQuest™ software. In each sample, a minimum of 20 000 cells were measured and the data were saved in the list mode. WinMDI™ software (version 2.8, Joseph Trotter, ScrippsResearch Institute, LaJolla, CA, USA) was used for off-line data processing. Gating was based on light scatter characteristics.

Lymphocyte transformation test

The activity of splenic lymphocytes was determined using the lymphocyte transformation test. For the test, the density of the cell suspension was adjusted to 1×10^6 per ml of RPMI 1640 medium (Sigma, Germany) supplemented with 10% precolostral calf serum, antibiotics (100 000 IU/l penicillin and 0.2 g/l streptomycin), and 2-mercaptho-ethanol (Fluka, Switzerland; 3.4 µl of a one thousand-times diluted solution into 1 ml of the cell suspension). The volume of 200 µl of the suspension was transferred into the wells in a 96-well flat-bottomed microtiter plate. Phytohaemagglutinin (PHA) at a concentration of 40 µg/ml was used for stimulation and all samples were run in triplicates. The microplates were incubated at 37°C under a 5% CO_2 atmosphere for three days. Twenty hours before harvesting (FilterMate Harvestor, Packard Bioscience Company), 50 µl of the medium with ^{3}H-thymidine (5 µCi/ml) was added. The incorporation of ^{3}H-thymidine was detected using a microplate scintillation and luminescence counter (TopCount NXT™, Packard Bioscience Company). The stimulation index (S.I.) was calculated as the ratio of counts per minute (CPM) in stimulated samples and in non-stimulated controls.

Cytokine gene expression

Ten million isolated splenocytes were diluted in 2 ml of RPMI 1640 supplemented with 5% bovine foetal serum, 100 000 IU/l of penicillin, and 0.2 g/l of streptomycin. Cells were stimulated for 4 h with PMA (50 ng/ml) (Serva, Germany) + ionomycin (2 µg/ml) (Sigma, Germany) at 37°C in an atmosphere containing 5% CO_2.

The total RNA was isolated using a Tri Reagent (MRC, Cincinnati, USA). Reverse transcription was carried out using the oligoT primer and AMV reverse transcriptase (ABgene, Epsom, UK). Primers and probes for the 5´ nuclease assay were adopted from Krook et al. (2002). The expression of IFN-γ and IL-4 was measured in duplex reactions with β-actin as a reference gene. The gene β-actin was selected from GAPDH and β-2 microglobulin due to its stable expression in treated cells and good amplification efficiency (data not shown). The Dynamo Probe qPCR Kit (Finnzymes, Espoo, Finland) with 0.8 pmol/µl for the final concentration of each primer, 0.1 pmol/µl for the final concentration of probes for IFN-γ and IL-4 and 0.2 pmol/µl for the final concentration of the probe for β-actin were used for real-time PCR on the LightCycler 1.2 (Roche, Mannheim, Germany). Cycling conditions were as follows: initial denaturation at 95°C for 15 min, then 50 cycles at 95°C for 15 s and at 58°C for 30 s. Probes for the expression of IFN-γ and IL-4 were labelled with FAM and BHQ1, the probe for β-actin with PULSAR and BHQ2 (Generi Biotech, Hradec Kralove, Czech Republic). Samples were run in duplicates. Blank control samples (no cDNA) and RNA isolation controls for genomic DNA contamination (total RNA without reverse transcription) were used. Amplification efficiency (E) for each gene was measured by serial dilutions of cDNA and used when calculating the relative expression of the gene of interest (goi) as a ratio of goi/ref. The relative expression at a mRNA level for each gene in each sample, as a portion of reference gene value, is prerequisite for the simple statistical comparison of different groups of outbred animals. The formula used: $goi/ref = [1/(Egoi^{Ctgoi})]/[1/(Eref^{Ctref})]$ is an alternative to the $2^{-\Delta Ct}$ method (Livak and Schmittgen, 2001), which means that amplification efficiencies for both genes can be counted on. Performing duplex reactions of the reference gene (β-actin) with IFN-γ or IL-4 significantly reduces pipetting errors and amplification inconsistencies and thus allows reliable measurements of gene expression to be made.

Statistical analysis

The statistical analysis of differences among groups of animals was performed by the nonparametric Kruskal-Wallis one-way ANOVA. In the

case of significant differences among groups ($P < 0.05$), the statistical analysis of differences between each two groups of animals was performed by the nonparametric Mann-Whitney test. In this case, the significant difference between groups was determined at $P < 0.05$. All statistic analyses were performed using Prism 3.03 software (GraphPad Software, San Diego, CA, USA).

RESULTS

Body weight

The body weights of 23 days old rats vary from 44.0 g to 64.5 g. Significant differences in body weight among groups were observed, animals from the group treated with 10.0 mg/kg of B[a]P were significantly lighter than the controls (Table 1).

Haematological analysis

Total counts of erythrocytes varied significantly from 3.6–4.5 × 10^{12} per litre. In animals from B[a]P-treated groups, the total erythrocyte counts decreased in a dose-dependent manner and were significantly lower in the 1.0 mg/kg and 10.0 mg/kg of B[a]P groups (Table 1).

Total leukocyte counts varied significantly from 3.4–5.6 × 10^{9} per litre. In B[a]P-treated groups, the leukocyte numbers differed from those found in the controls. A significantly lower number of leukocytes was found in the group treated with 1.0 mg/kg of B[a]P (Table 1). Differential counts did not differ among the groups and varied within the following ranges: lymphocytes 66.5–85.0%, neutrophils 14.0–28.5%, monocytes 1.0–5.2% and eosinophils 0.0–1.2 %.

Determination of lymphocyte subsets

Samples of spleen obtained from the B[a]P-treated rats contained significantly higher percentages of T lymphocytes characterized by the expression of either CD4+ or CD8+ than those obtained from the controls (Table 1). A significantly higher number of both (CD4+ or CD8+) types of lymphocytes was counted in the group treated with 0.1 mg/kg of B[a]P. The proportions of γδ+ TCR lymphocytes and B lymphocytes (defined as sIgM-positive cells) among groups were not significantly different (data not shown).

Lymphocyte transformation test

When compared with the controls, the mitogenic activity of B[a]P-treated animals was higher (Table 1). Results of the test are expressed in terms of a spontaneous and PHA-induced ^{3}H-thymidine

Table 1. Effects of postnatal exposure (PND 1–14) to benzo[a]pyrene on the immunity of immature rats (PND 23)

	Control	B[a]P		
		0.1 mg/kg	1.0 mg/kg	10.0 mg/kg
Body weight (g)	56.29 (0.53)	59.34 (0.99)	55.12 (1.94)	47.21 (1.48)***
Erythrocytes (10^{12}/l)	4.17 (0.09)	3.92 (0.21)	2.83 (0.15)***	3.02 (0.24)**
Leukocytes (10^{9}/l)	4 440 (224)	5 060 (432)	3 660 (287)*	6 185 (723)
CD4+ splenocytes (%)	18.41 (1.44)	24.10 (0.97)**	19.57 (0.88)	22.14 (1.65)
CD8+ splenocytes (%)	16.49 (1.42)	21.62 (0.78)*	19.18 (0.75)	15.59 (1.30)
Spontaneous mitogenic activity (CPM)	265 (42)	201 (14)	449 (54)*	413 (67)
PHA stimulated mitogenic activity (CPM)	589 (109)	1 091 (198)*	2 301 (430)***	1 596 (339)*
IL4 (IL4/β-actin ratio)	0.053 (0.005)	0.030 (0.003)**	0.033 (0.002)**	0.028 (0.005)**
IFNγ (IFNγ/β-actin ratio)	1.168 (0.124)	0.898 (0.045)	0.850 (0.067)	0.397 (0.082)***
IFNγ/IL4 ratio	22.20 (1.12)	33.13 (2.88)**	26.30 (1.82)	16.43 (3.61)*

PND = postnatal day; CPM = counts per minute

Data are presented as mean and standard error of the mean (SEM). Groups significantly different in comparison to the control are marked with asterisks: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

incorporation. Subsequently, the stimulation indexes were calculated. In contrast to the stimulation indexes, where no differences were found, the spontaneous mitogenic activity and mitogenic activity after PHA induction were significantly different among individual groups. As compared with the controls, the spontaneous mitogenic activity was significantly higher in the group receiving 1.0 mg/kg of B[a]P while the PHA-induced mitogenic activity was significantly higher in all treated groups.

Cytokine gene expression

Cytokine gene expression of IL-4 and IFN-γ, as well as their ratio significantly varied among groups of animals. The relative expression of IL-4 and IFN-γ was described as a ratio to the β-actin gene. When compared with the control, all groups of B[a]P-treated animals showed a similar significant decrease in the expression of IL-4 (Table 1). There was no significant difference among individual B[a]P-treated groups. The expression of IFN-γ was also decreased in all B[a]P-treated groups but only the group with the highest dose of B[a]P showed a significant difference compared to the control. It was found that, when compared with the control, the IFN-γ/IL-4 ratio was significantly increased in the group receiving 0.1 mg/kg of B[a]P while in the group with the highest (10.0 mg/kg) dose of B[a]P it was significantly decreased. In the group receiving 1.0 mg/kg of B[a]P the IFN-γ/IL-4 ratio was not significantly different from the control (Figure 1).

DISCUSSION

This study is the first attempt to determine the effect of different doses of B[a]P on the developing immune system of immature rats. Based on the results obtained it was possible to conclude that many parameters under study were influenced. The only non-affected parameters were the percentages of splenic γδ+ TCR lymphocytes and B lymphocytes and the differential counts of blood leukocytes.

The decreasing total counts of erythrocytes in the peripheral blood of animals treated with 1.0 mg/kg

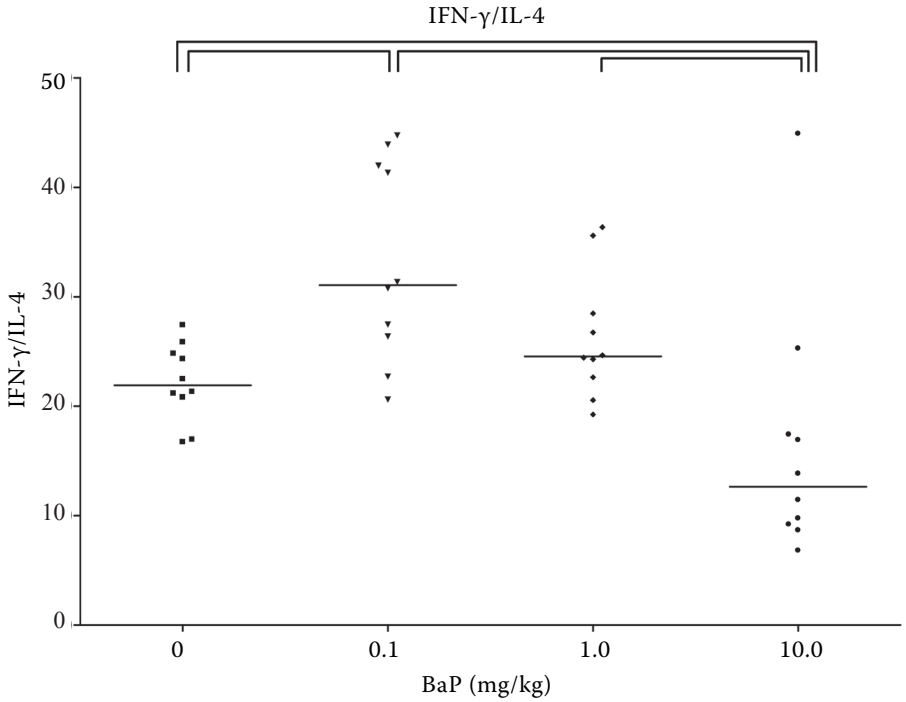

Figure 1. Results of real-time PCR detection – the IFN-γ/IL-4 mRNA ratio in lymphocytes obtained from the spleen of immature rats (PND 23) postnatally (PND 1–14) exposed to benzo[a]pyrene at the concentration 0; 0.1; 1.0 and 10.0 mg/kg

PND = postnatal day

Data are presented as individual results and median. Groups significantly different ($P < 0.05$) are connected by the lines

and 10.0 mg/kg of B[a]P could indicate that B[a]P has a detrimental effect on bone marrow erythropoiesis. This was probably caused by an apoptosis of red blood cell progenitors induced by the genotoxic effects of B[a]P (Ko et al., 2004) and/or its metabolites on bone marrow (Yoon et al., 2001). The decrease in numbers of erythrocytes was associated with decreased body weight because the animals in the group receiving 10.0 mg/kg of B[a]P were significantly lighter. de Jong et al. (1999) also observed a dose-dependent decrease in counts of red blood cells associated with subtle changes in body weight in mature rats after an oral administration of different doses of B[a]P.

The decrease in numbers of erythrocytes was not correlated with the total numbers of leukocytes because in the peripheral blood of B[a]P-treated animals these numbers were similar to those in the control group. When compared with all other groups, only the animals receiving 1.0 mg/kg of B[a]P showed a significant decrease in counts of leukocytes. Changes in numbers of lymphocytes, neutrophils, monocytes and eosinophils in blood were not significant due to high variances within individual groups (data not shown), so that it was not possible to determine the cause of leukocytes decrease in group exposed to 1.0 mg/kg of B[a]P. It was not probable that this could be caused by T lymphocytes decrease as in the spleen of this group both the CD4+ and CD8+ T lymphocytes were at the same level as in the control. Moreover, when compared with the control, a significant increase in splenic T lymphocytes CD4+ and/or CD8+ counts was observed in the group exposed to the lowest dose (0.1 mg/kg) of B[a]P while in that with the highest dose these counts were not different. Jong et al. (1999) found that a decrease in levels of leukocytes in the peripheral blood in adult rats exposed to a high dose of B[a]P was caused by the depletion of lymphocytes and eosinophilic granulocytes. One of the possible reasons of the decreased counts of lymphocytes could be the induction of apoptosis by B[a]P and its metabolites in pre-B cells and in B lymphocytes (Salas and Burchiel, 1998). However, when sIgM-positive cells in the spleen were measured, no significant changes were observed due to the existence of significant variances within individual groups (data not shown). In a study performed with adult rats (de Jong et al., 1999) a significant decrease in numbers of splenic B lymphocytes was found.

Our findings that the *in vivo* exposure to B[a]P increases the spontaneous mitogenic activity of isolated immature rat splenocytes as well as the mitogenic activity after PHA stimulation is in agreement with the reported increased mitogenic activity of adult mouse PHA stimulated splenocytes after exposure to B[a]P *in vitro* (Burchiel et al., 2004). On the other hand, the mitogenic activity of human-PHA-stimulated peripheral lymphocytes after exposure to B[a]P *in vitro* was decreased (Davila et al., 1996).

Despite the increased mitogenic activity of isolated rat splenocytes, the mRNA levels of IL-4 decreased in a similar manner in all B[a]P-treated groups. A similar effect on isolated human basophiles was also observed (Kepley et al., 2003). The decreasing mRNA level of IFN-γ only in the group of animals treated with the highest dose of B[a]P implies a lower sensitivity of the Th1 signaling pathway to B[a]P then the Th2 one. This is well illustrated in Figure 1, where the ratios of IFN-γ/IL-4 are presented. The lowest dose of B[a]P (0.1 mg/kg) significantly increased the IFN-γ/IL-4 ratio (a shift to the Th1 type immune response). A shift to the Th1 response (increasing IFN-γ) was also observed in splenocytes isolated from adult mice exposed to diesel exhausts *in vivo* (Fujimaki et al., 2001). The ability to modulate Th1/Th2 immune response was also observed in PAHs containing diesel exhaust (van Zijverden et al., 2000). When compared with controls, the highest dose of B[a]P significantly decreased the IFN-γ/IL-4 ratio (a shift to the Th2 type immune response). The Th2 shift in the group with the highest dose of B[a]P through the estrogenic activity of this PAH was also possible as estrogen alone may have this effect (Huber et al., 1999; Salem, 2004). Several recent studies have reported that some PAHs, including B[a]P can activate estrogen receptors, either directly or indirectly by producing estrogenic metabolites (Charles et al., 2000; Fertuck et al., 2001; Pliskova et al., 2005).

Based on data presented in this study it can be hypothesized that the early exposure of developing animals to PAHs might have an impact on the modulation of their immune response to infection and/or vaccination.

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Corresponding Author:

Jan Matiasovic, Veterinary Research Institute, Hudcova 70, 621 00, Brno, Czech Republic
Tel. +420 533 331 317, fax +420 541 211 229, e-mail: matiasovic@vri.cz