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MODULATION OF T-CELL ONTOGENY BY TRANSPLACENTAL BENZO(a)PYRENE

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Abstract — Transplacental exposure to the carcinogen, benzo(a)pyrene BaP, leads to depressed immune function and increased tumor incidence in mice. This paper reports ontogenetic T-cell changes in BALB/c mice after exposure to BaP *in utero*. Monoclonal antibodies (MAbs) were produced to fetal liver T-cells (FLT) and newborn spleen (NBS) lymphocytes purified from offspring of pregnant BALB/c mice that were given one injection of BaP (150 mg/kg body weight) in mid-gestation (day 11–13). The MAbs reacted with two T-cell membrane antigens (FLT and NBS) found in fetal liver, neonatal and adult thymus and spleen. Lymphocytes of BaP-exposed 19-day fetuses showed decreased subpopulation frequencies ($P<0.05$) in fetal liver total *T*-cells (from 56% to 16%), Ly1 cells (from 33% to 9%), and Ly2 cells (from 56% to 1%) compared with untreated controls. In contrast, BaP increased the subpopulation frequencies ($P<0.05$) in FLT cells in fetal liver (from 20% to 52%) and in newborn spleen (from 21% to 51%), and increased NBS cells in newborn spleen (from 24% to 59%). The increased frequency in FLT and NBS cells was due to their relative resistance to BaP toxicity and/or BaP-enhanced proliferation in the neonatal period. Compared with untreated controls, BaP treatment resulted in reduced numbers of T-cells in fetal liver and showed a selective toxicity for Ly1 cells (89% reduction) and Ly2 cells (99% reduction), whereas FLT cells were not reduced and NBS cells were reduced by 60%. Six-week-old juvenile mice exposed to BaP *in utero* showed recovery of total T-cells to control levels in spleen and thymus, but showed depletion ($P<0.01$) in thymic FLT cells (from 81% to 12%) and in splenic NBS cells (from 55% to 16%). The monoclonal antibodies developed for this study recognize novel cellular changes in the murine immune system that are associated with transplacental BaP. The FLT and NBS antigens may be useful biomarkers for developmental immune dysfunctions in progeny exposed to BaP *in utero*.

Keywords: murine fetal T-cells, T-cell antigens, benzo(a)pyrene.

While polycyclic aromatic hydrocarbons (PAH) are best known for carcinogenic activity, they also exhibit immunosuppressive activities (Luster & Blank, 1987). Suppression of humoral and cellular immune functions has been demonstrated in adult mice by *in vivo* (Stjernsward, 1966; Dean *et al.*, 1983; Ward *et al.*, 1984; Wojdani & Alfred, 1984; White *et al.*, 1985; Lyte & Bick, 1986; Blanton *et al.*, 1986; Gruffe *et al.*, 1986; Meyers *et al.*, 1987) as well as *in vitro* exposure to PAH, including benzo(a)pyrene (BaP), benzanthracene, and methylcholanthrene (Alfred & Wojdani, 1983; Urso *et al.*, 1986; Ghoneum *et al.*, 1987; Kawabata & White, 1987). In the developing fetus, BaP is a transplacental carcinogen (Bulay & Wattenberg, 1970; Nikinova, 1977; Urso & Gengozian, 1980, 1982), embryotoxin and teratogen (Rigdon & Renneis, 1964; Lambert & Nebert, 1977; Shumm *et al.*, 1979; Barbieri *et al.*, 1986). Progeny exposed to BaP *in utero* during mid-gestation have also shown long-lasting humoral and cellular immune dysfunction (Urso & Gengozian, 1980, 1982,

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Abbreviations — BaP, Benzo(a)pyrene; CO, corn oil; D-PBS, Dulbecco's phosphate buffered saline; FLT, fetal liver T-cells; HBSS, Hank's balanced salt solution; MAb, monoclonal antibody; PCFIA, particle concentration fluorescence immunoassay; NBS, newborn spleen cells; NBT, newborn thymocytes; pristane, 2, 6, 10, 14-tetramethylpentadecane.

1984; Urso & Johnson, 1987; Bast *et al.*, 1980). Increased susceptibility of C3H murine offspring to lung and liver tumors, after transplacental BaP exposure, has been associated with changes in the relative proportions of Lyt T-cell differentiation markers (Urso & Johnson, 1987).

The mechanisms responsible for immunotoxic effects by BaP are not known. BaP is a procarcinogen that is metabolically oxidized to the chemically reactive species, BP-7, 8-diol-9, 10-epoxide (BPDE). DNA damage occurs when electrophilic BPDE binds covalently to nucleophilic DNA (Lambert & Nebert, 1977; Shumm *et al.*, 1979). Increased metabolic activation of BaP via induction of aryl hydrocarbon hydrolase has been shown to occur in mouse (Shumm *et al.*, 1979), guinea-pig (Bast *et al.*, 1980) and human lymphocytes (Whitlock *et al.*, 1972; Boobis *et al.*, 1978). BPDE-DNA adducts have been demonstrated in peripheral blood lymphocytes of coke oven workers exposed to high levels of BaP (Harris *et al.*, 1985; Haugen *et al.*, 1986). Clonal deletions or anergy could arise from genotoxic effects of BPDE in developing lymphocytes. BPDE also forms adducts with proteins (Shugart & Malsunami, 1985), and alterations in Class II MHC (Ia) antigens affecting antigen presentation have been implicated in BaP-induced immunosuppression (Meyers *et al.*, 1987). Alternatively, the tumorigenic and toxic effects of BaP for a variety of cell types could result in downregulation of immunocytes by cytokine mediators (Gruffe *et al.*, 1986; Ghoneum *et al.*, 1987) that influence proliferation and differentiation pathways in the immune system.

In this study, modulation of lymphocyte Lyt differentiation antigens by transplacental BaP has been analyzed in BALB/c mice. BaP modulation of additional ontogenetic changes in perinatal lymphocytes has been addressed through the use of novel syngeneic monoclonal antibodies produced to BaP-exposed lymphocyte subpopulations present in fetal liver and newborn spleen. The monoclonal antibodies generated appear to recognize markers of BaP-resistant T-cells, that persist independently of Lyt antigen expression. The incidence and frequency of perinatal cells expressing these antigens has been compared in transplacental BaP-exposed and non-exposed mice.

EXPERIMENTAL PROCEDURES

Animals

Twelve male and twenty-four female BALB/c mice from The Jackson Laboratory (Bar Harbor, ME, U.S.A.) were bred in-house (AAALAC-accredited animal facility) to obtain progeny for study. Females were used at 6–10 weeks of age. The colony of mice was periodically tested to ensure that the animals remained specific-pathogen-free. Commercial rodent chow (Purina) was fed *ad libitum*. Mice were housed in polycarbonate cages with sterilized hardwood chips at four to six animals per cage. Purified water was provided by water bottles. Light–dark cycles were controlled on a 12 h on–off routine. Temperature and humidity were kept at an average of 72°F and 45%, respectively.

Benzo(a)pyrene treatment of mice

Male and female virgin mice were mated for 1–4 days, and insemination was verified by the appearance of a vaginal plug. About 80% of inseminated females became pregnant. Pregnant females were injected once with 150 mg/kg of BaP (Aldrich Chemicals) dissolved in corn oil (Kodak, Lot #A14A, maximum free fatty acid content of 0.04 mEq, iodine value 102–128) or with corn oil vehicle only (controls) between days 11 and 13 of gestation (17). Treatment of non-pregnant female controls or male mice of similar age with BaP followed the same time schedules used for pregnant mice.

Lymphoid cell preparations

Fetal liver and thymus were collected from the fetuses of sacrificed pregnant mice between days 17 and 19 of gestation. Newborn spleen and thymus were obtained between days 3 and 5 post-partum. Fetal or neonatal tissues from the offspring of three to five females per group were pooled to obtain a sufficient quantity of cells for study. The thymus and spleen from groups of six juvenile mice were obtained and individually analyzed at 3 weeks and at 6 weeks after birth. Maternal tissues were obtained from pregnant females sacrificed to obtain fetal tissues. Control non-pregnant female or male mice were sacrificed 3–6 days following BaP or corn oil treatments. Cell suspensions were prepared by gently pressing tissues in 10 ml Hank's Balanced Salt Solution (HBSS) through sterile wire mesh strainers. Cells were washed twice with cold HBSS, and adherent cell types were removed by plating cell suspensions in plastic Petri dishes for 2 h at 37°C. Lymphocytes were purified by density gradient centrifugation using Lympholyte Medium for mouse lymphocytes (Cedarlane Laboratories, Accurate Scientific Corp.), and washed twice with HBSS. Total cell numbers and cell viabilities were determined by the Trypan Blue exclusion test in a hemacytometer counting chamber.

Hybridization of spleen cells

Adult male BALB/c mice were immunized i.p. with 96% viable purified lymphocytes from tissues obtained from progeny exposed to BaP *in utero*, with either 2×10^6 fetal liver T-cells (FLT MAb) or 1×10^7 newborn spleen cells (NBS MAb) suspended in 0.1 ml HBSS that had been emulsified in an equal volume of complete Freund's adjuvant (Gibco Scientific). Four weeks after immunization, mice were bled from the tail vein to obtain sera for antibody testing, then mice were restimulated with 5×10^6 viable cells (either FLT or NBS as before) in HBSS. Three days later, spleen cells from the immunized mice were harvested and hybridized to X63.653.Ag1 myeloma cells (Oi & Herzenberg, 1980) for evaluation of the production of specific monoclonal antibodies (MAb) to T-lymphocytes.

Screening and selection of hybridomas

Supernatants of hybrid clones were screened at 4 weeks post-hybridization for MAb by indirect ELISA tests using glutaraldehyde-fixed cells in flat-bottom 96-well microtiter plates. Sterile ELISA plates (Dynatech Easywash) were pretreated with poly-L-lysine (100 μg/ml in deionized H_2O, 70–150 kDa, 0.1 ml/well) to enhance lymphocyte adherence. Lymphocytes were prepared at 2×10^6 cells/ml in Dulbecco's PBS (D-PBS) and 0.1 ml of cells were added to each well. Cells were allowed to settle overnight at 4°C, and fixed to the wells by adding 20 μl of 2.5% glutaraldehyde in D-PBS for 20 min. Plates were washed 4 times with D-PBS, filled with D-PBS, 0.1% bovine serum albumin for blocking, then the plates were sealed and stored overnight at 4°C. Screening of hybrid cell supernatants was accomplished by adding 0.1 ml of cell supernatant to fixed, washed cells, and performing an indirect ELISA test using alkaline phosphatase conjugated goat anti-mouse IgM, Fc-specific (Sigma Chemical Co.). Plate washes were performed with 0.3 ml/well PBS, 0.05% Tween 20 (using a Dynatech 96-well plate washer), and readings were made at 410 nm (Dynatech MR 700 dual-beam ELISA plate reader).

Antibody isotypes of hybridomas were determined by gel diffusion analysis of cell supernatants versus antisera specific for mouse IgM, IgA, IgG_1, IgG_{2a}, IgG_{2b}, and IgG_3 (Miles Laboratories). All MAb to lymphocytes identified were of the IgM isotype.

Immunofluorescence analysis of antibody reactions was carried out by adding 10 μl of lymphoid cells (2×10^6/ml) to glass slides, allowing cells to dry for 1 h at room temperature, then fixing cells by placing slides in 98% ethanol for 10 min, and allowing to air dry. Indirect immunofluorescence was performed by adding 50 μl of hybridoma supernatant to the fixed cell smear for 1 h at room temperature, washing for 1 h at room temperature with PBS, adding 50 μl of fluorescein conjugated goat anti-mouse IgM (Sigma Chemical Co.) for 1 h at room temperature, then washing 1 h with PBS. Cells were mounted under coverslips with 90% glycerol, 10% PBS and readings were performed with a Nikon Diaphot microscope equipped for epifluorescence. All positive clones showed ring fluorescence, indicative of membrane antigen localization.

Hybridomas were established as transplantable ascites tumors by pretreatment of BALB/c mice with i.p. injection of 0.5 ml pristane (Aldrich Chemical Co.). Between 7 and 30 days later, mice were injected with 1×10^6 hybridoma cells. Ascites fluid was removed from mice that developed tumors and was transplanted to other mice or was diluted 1:2 in HBSS for use as an *in vitro* source of monoclonal antibody.

Cytotoxicity assay

Purified viable lymphocytes were obtained from lymphoid tissues and internally labeled with the fluorescent dye, 5(and-6)-carboxy-2', 7'-dichlorofluorescein diacetate succinimidyl ester (CF-1165, Molecular Probes, Inc.). The dye was dissolved in dimethylsulfoxide to 10 mg/ml and diluted to 33 μg/ml in D-PBS. Cells in D-PBS at 2×10^6/ml were mixed with dye at a 5:2 cell:dye volumetric ratio, incubated for 30 min at 37°C, washed twice with D-PBS, and resuspended to 2×10^6/ml in cytotoxicity medium (RPMI containing 10% crystalline BSA). Twenty microliters containing 10,000 labeled cells were incubated with 20 μl of MAb for 15 min, then 20 μl of Low-Tox™-M rabbit complement (Cedarlane Laboratories), diluted 1:5 in HBSS were added and reactions were incubated for 1 h at 37°C. The number of viable cells was determined by fluorescence readings of washed, packed cells using a Pandex PCFIA system. The relative numbers of viable cells remaining after the reaction were determined for cells treated with antibody + complement (test cells) or with complement alone (control cells). The percentage of lymphocytes expressing cell membrane antigen was determined from the mean fluorescence reading of quadruplicate tests by calculating the cytotoxic index (percentage cytotoxicity) from the formula:

$$\%C = \frac{\text{control cells} - \text{test cells}}{\text{control cells}} \times 100.$$

Table 1. Reactions of lymphoid cells with syngeneic serum antibodies produced to BaP-exposed fetal and newborn lymphocytes*

Antiserum: Cell type‡	Cytotoxic index†					
	Ly1.2	Ly2.2	BaPFL#1	BaPFL#2	BaPNBS#1	BaPNBS#2
BaP-fetal liver	9	1	11	0	11	14
BaP-fetal thymus	80	18	46	52	44	94
BaP-maternal spleen	74	90	21	12	0	14
Normal paternal spleen	52	49	5	0	0	20
BaP-paternal spleen	64	51	13	2	18	26
Normal paternal thymus	57	45	43	0	55	45
BaP-paternal thymus	91	58	36	32	54	52

†Lymphoid cell populations were obtained from untreated and BaP-treated male, gestating female mice and BaP-exposed fetal liver or thymus and analyzed for reactions with antisera to Lyt antigens and with serum obtained from BALB/c mice immunized with BaP-exposed fetal liver cells (BaPFLT #1 and #2) or with BaP-exposed newborn spleen cells (BaPNBS #1 and #2).

†The relative numbers of cells expressing antigens were determined as the percentage of cells killed by antibody plus complement (cytotoxic index).

‡Two pregnant females and one paternal male were treated with 150 mg/kg benzo(a)pyrene on day 12 of pregnancy. Mice were sacrificed on day 18 of pregnancy to obtain lymphoid tissues.

Antisera

Rabbit anti-mouse lymphocytic serum (ALS) and rabbit anti-mouse thymocyte serum (ATS) were obtained from Accurate Chemical and Scientific Corporation and used at a final dilution of 1:300. Anti-Ly1.2 and anti-Ly2.2 alloantisera were obtained from Cedarlane Laboratories Ltd and used at a final dilution of 1:30. Ascites fluid containing MAb to the FLT antigen was used at a 1:100 dilution. Hybridoma culture supernatant containing MAb to the NBS antigen was used at a 1:10 dilution.

Statistics

Cytotoxicity data were analyzed by the *t*-test for two independent means, and differences in treatment groups were considered significant when the two-tailed *P* values were ≤ 0.05. Each data set consisted of the mean values obtained from three to six independent experiments, in which the average cell frequencies were obtained from four replicate determinations.

RESULTS

Production and screening of monoclonal antibodies

Immunized BALB/c mice produced a serum antibody reactive with syngeneic adult male and female lymphocytes as well as with BaP-exposed fetal cells. The BALB/c immune sera showed antibody reactions that were comparable with the reactions observed with allogeneic antisera to BALB/c Lyt antigens (Table 1). The greatest number of cells expressing antigens recognized by the mouse antisera was found in fetal thymus, followed by normal adult thymus.

Hybridization of spleen cells yielded 61 hybridomas, all producing IgM antibody against target cell antigens (fetal liver cells, newborn spleen cells, newborn thymocytes, maternal spleen cells, or adult male spleen cells from mice that were treated with either benzopyrene or corn oil) when screened by ELISA tests. The MAbs showed reactions with both BaP-exposed and non-BaP-exposed tissues; hence no MAb was detected with any apparent specificity for the BaP chemical group (moiety or adduct).

ELISA titration of MAbs

A MAb produced by a hydridoma derived from the spleen cells of BaPFL#1 mouse was used for studies of anti-fetal liver T-cell specificity (MAb FLT). Studies of anti-newborn spleen T-cell specificity were carried out using MAb NBS from a hybridoma derived from the spleen cells of BaPNBS#2 mouse. ELISA titrations of the antibody activities of MAbs FLT and NBS for corn oil control or BaP-exposed fetal liver cells and newborn spleen cells are shown in Fig. 1A–D. Both antibodies showed high binding activity for glutaraldehyde fixed cells. The ELISA reagent was an anti-mouse IgM conjugate that could also have reacted with B-cells expressing sIgM (surface membrane-bound immunoglo-

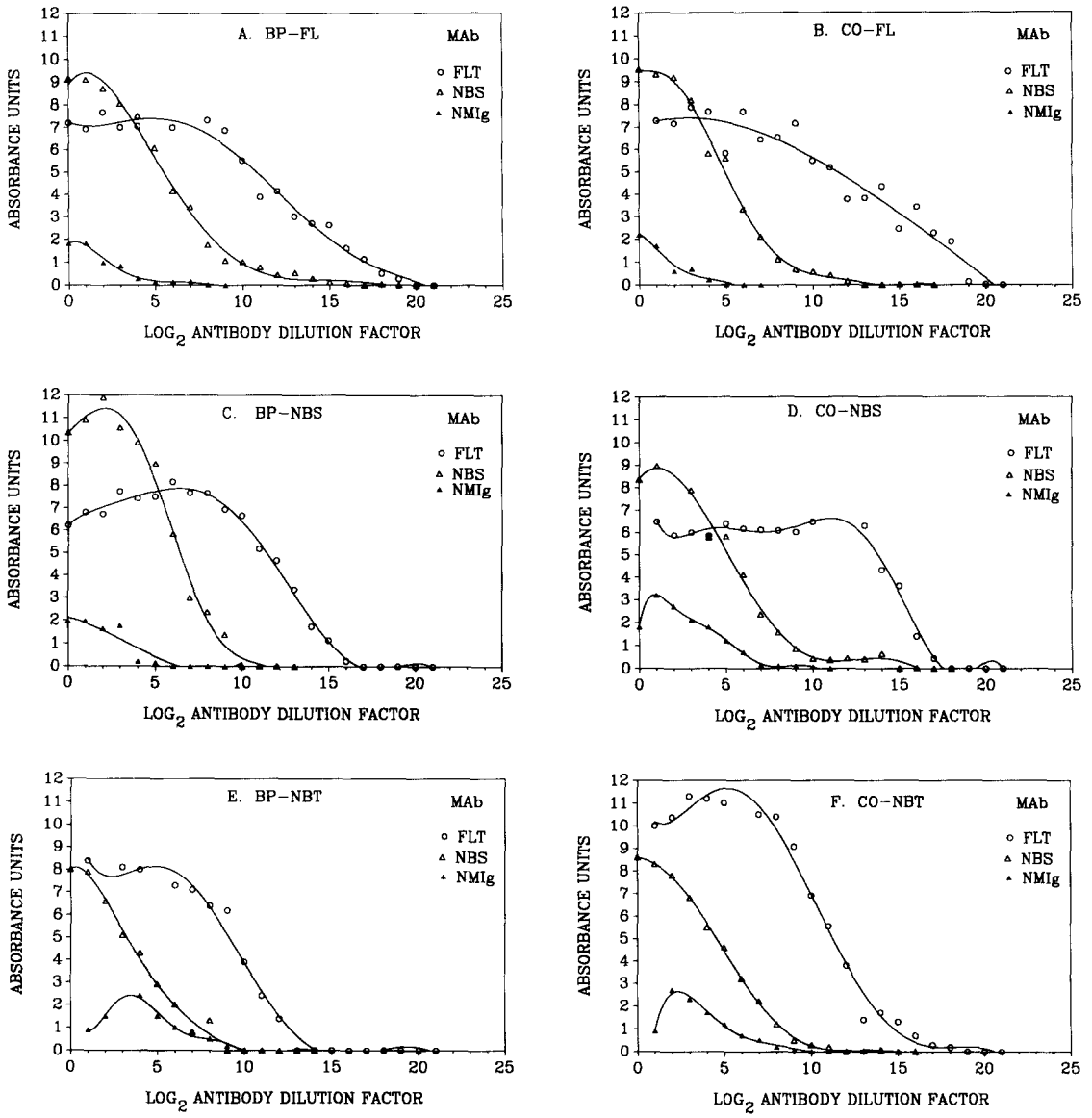

Fig. 1. Titrations of monoclonal antibody activities by ELISA. Absorbance units are the optical density readings at 410 nm of 10-fold dilutions of microtiter plate well contents, multiplied by the dilution factor. Antibodies were titrated versus: (A) BP-FL, benzopyrene-exposed fetal liver cells; (B) CO-FL, corn oil-treated (control) mouse fetal liver; (C) BP-NBS, transplacental benzopyrene-exposed newborn spleen cells; (D) CO-NBS, control newborn spleen cells. (E) BP-NBT, transplacental benzopyrene-exposed newborn thymocytes; (F) CO-NBT, control newborn thymocytes. Antibodies tested were MAb FLT, produced to BaP-exposed fetal liver lymphocytes; MAb NBS, produced to BaP-exposed newborn spleen lymphocytes; NMIg, normal mouse immunoglobulins, used as the control for non-specific immunoglobulin binding to cells.

bulin). The low background reactions with normal mouse immunoglobulins (used as the control for non-specific binding by mouse monoclonals) show that the reactions were not due to B-lymphocytes in the neonatal tissues tested. Since the MAbs reacted with purified thymocytes (Fig. 1E–F), it was concluded that the MAbs recognized epitopes associated with normal T-cell antigens.

Table 2. Normal frequencies of lymphoid cell subpopulations in BALB/c mice

Tissue	Percentage of cells expressing antigen* (Mean ± S.D.) Ly1	Ly2	FLT	NBS
Fetal liver	32.8 ± 1	55.5 ± 16	20.3 ± 14	72.9 ± 3
Fetal thymus	14.2 ± 8	28.4 ± 6	24.1 ± 19	38.4 ± 16
Newborn spleen	65.0 ± 19	65.8 ± 29	21.3 ± 8	24.4 ± 3
Newborn thymus	60.2 ± 13	57.7 ± 20	34.5 ± 10	40.1 ± 21
Juvenile spleen	ND†	ND†	29.9 ± 26	54.9 ± 21
Juvenile thymus	ND†	ND†	84.0 ± 18	32.1 ± 19
Adult spleen	51.8 ± 14	49.1 ± 16	27.7 ± 15	61.0 ± 13
Adult thymus	69.9 ± 14	68.8 ± 12	30.3 ± 14	44.8 ± 21

*Values determined from the cytotoxic index.
†Not determined.

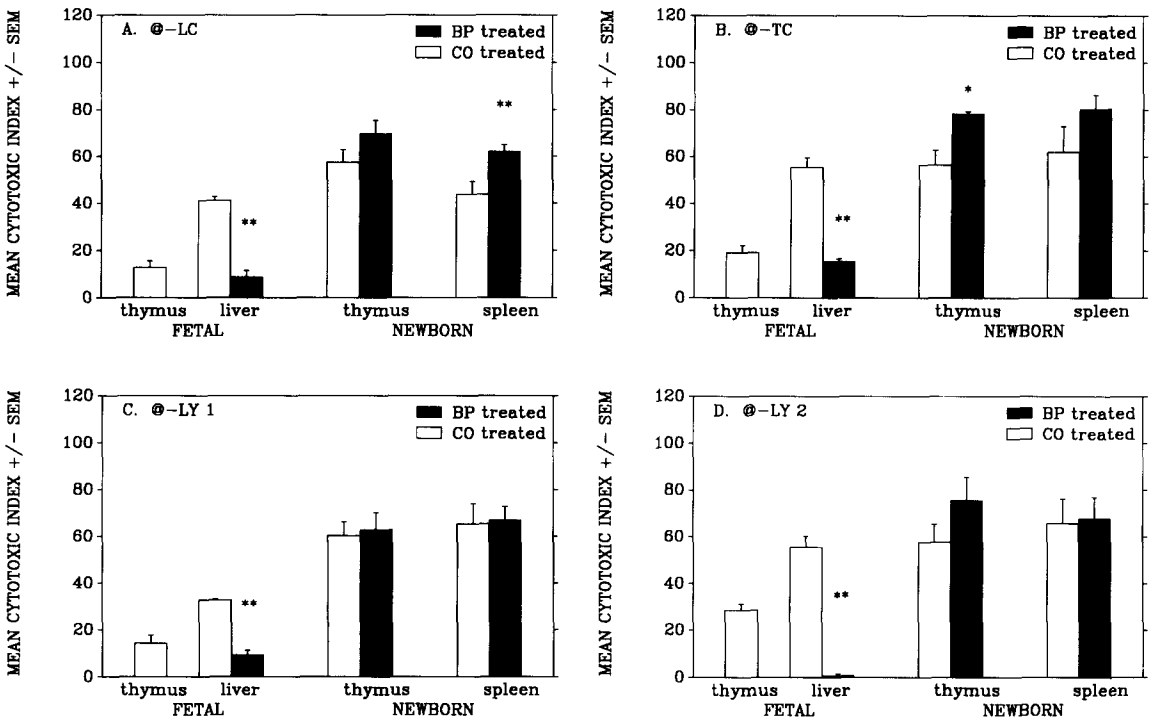

Fig. 2. Percentage of purified lymphocytes from control (CO treated) or BaP-exposed progeny (cytotoxic index), expressing antigen markers reactive with: (A) @-LC, antilymphocytic serum; (B) @-TC, anti-T-cell serum; (C) @-Ly1, anti-Ly1; (D) @-Ly2, anti-Ly2. The mean values obtained from three to six independent experiments are shown. Each experiment consisted of four replicate determinations on the pooled tissues from offspring of three to five mothers. Significant differences in CO-control and BaP-treatment groups are shown as $*P<0.05$, $**P<0.01$.

Membrane localization of FLT and NBS antigens

Immunofluorescence reactions of the MAbs with ethanol-fixed thymocytes and purified lymphocytes showed ring fluorescence, indicating that the antigen epitopes were associated with membrane bound antigens (data not shown). Complement-dependent cytotoxicity assays were used to determine the percent of lymphocytes that expressed antigens, and the results are presented below.

Normal frequencies of FLT+ and NBS+ cells in mice

The FLT and NBS antigens were found to occur in

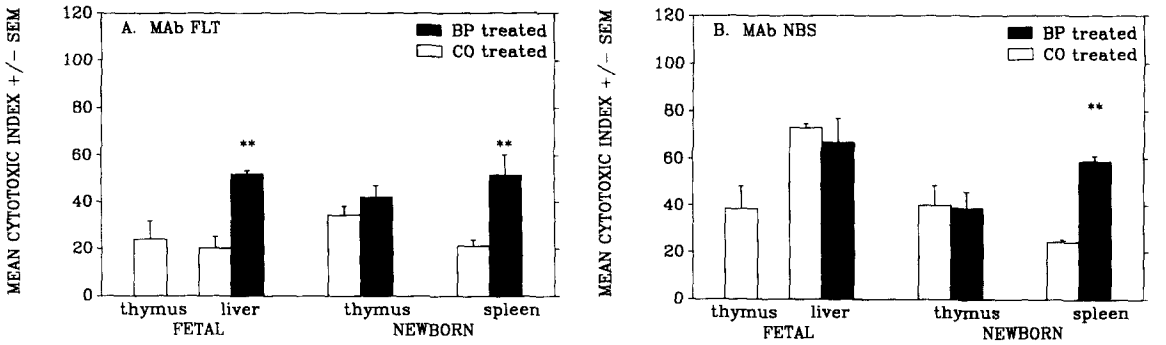

Fig. 3. Percentage of purified lymphocytes from control (CO treated) or BaP-treated progeny (cytotoxic index), expressing antigen markers reactive with: (A) MAb-FLT, produced to fetal liver lymphoid cells from BaP-treated progeny; (B) MAB-NBS, produced to splenic lymphoid cells from newborn progeny exposed to BaP *in utero*. The mean values obtained from six independent experiments are shown. Each experiment consisted of four replicate determinations on the pooled tissues from offspring of three to five mothers. Significant differences in CO-control and BaP-treatment groups are shown as $^{**}P<0.01$.

normal lymphoid cells of control fetal, newborn, juvenile and adult BALB/c mice. The relative frequencies of cells expressing FLT, NBS, Ly1, and Ly2 antigens in untreated BALB/c mice are shown in Table 2. The highest frequency of FLT+ cells (84%) was found in thymocytes from 6-week-old juvenile mice. High frequencies of NBS+ cells were found in fetal liver lymphocytes (73%) and adult spleen cells (61%). The age-related changes and organ distributions of these antigens suggest that they could be differentiation markers associated with different stages of T-cell maturation. The antigens were also found in spleen cells of adult C57BL/6 mice (23% FLT+; 28% NBS+).

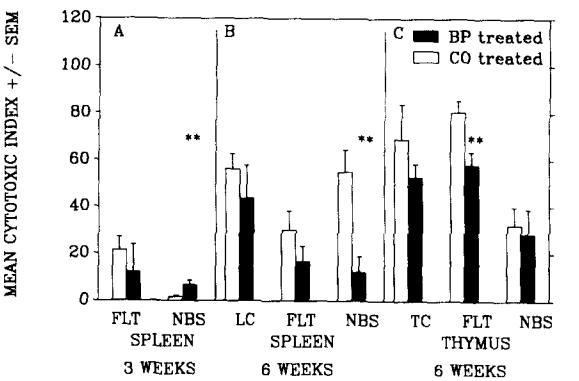

Fig. 4. Percentage of purified lymphocytes from juvenile mice, exposed to corn oil (CO-treated) or BaP (BP-treated) *in utero*, at 3 weeks or at 6 weeks of age (cytotoxic index), expressing antigen markers reactive with specific antibody: (A) FLT+ and NBS+ cells in splenic lymphocytes at 3 weeks; (B) ALS reactive cells (LC), FLT+, and NBS+ cells in splenic lymphocytes at 6 weeks; (C) ATS reactive cells (TC), FLT+, and NBS+ cells in thymocytes at 6 weeks. The mean values obtained from three to six independent experiments are shown. Each experiment consisted of four replicate determinations on the pooled tissues from the offspring of three to five mothers. Significant differences in CO-control and BaP-treatment groups are shown as $^{**}P<0.01$.

BaP-induced changes in the cellular frequencies of T-cells expressing Lyt differentiation antigens

The effect of transplacental BaP exposure on the relative frequencies of T-cells and Lyt subsets in fetal liver and newborn thymus and spleen lymphocyte populations is shown in Fig. 2A–D. In the fetal liver of corn oil controls, 56% of recovered lymphocytes expressed ATS T-cell antigen (ATS+, Fig. 2A), 56% were Ly2+ (Fig. 2D), and 33% were Ly1+ (Fig. 2C). BaP treatment resulted in severe depletions of ATS+ (15%, Fig. 2A) and both Ly1+ (9%, Fig. 2C) and Ly2+ (1%, Fig. 2D) subsets in fetal liver. Control fetal thymus contained 14% Ly1+ cells (Fig. 2C), and 28% Ly2+ cells (Fig. 2D). BaP treatment resulted in extreme atropy of fetal thymus and sufficient numbers of cells could not be recovered for analysis. In one experiment in which pooled BaP-exposed fetal thymocytes were analyzed by Trypan Blue exclusion, the cells were 80% Ly1+, 18% Ly2+ (Table 1).

In newborn mice, spleen and thymus cells from corn oil controls contained about 60% Ly1+ cells (Fig. 2C), 60% Ly2+ cells (Fig. 2D) and 60% ATS+ cells (Fig. 2B). Mice exposed to BaP *in utero* showed increases in ATS+ (80%, Fig. 2B) and thymic Ly2+ (76%, Fig. 2D) cells that were suggestive of Ly2+ cell activation.

BaP-induced changes in the cellular frequencies of T-cells expressing FLT and NBS antigens

The FLT MAb reacted with 24% of thymocytes, 20% of fetal liver cells, 35% newborn thymocytes, and 21% splenic lymphocytes in control progeny (Fig. 3A). BaP treatment *in utero* resulted in significant increases in fetal liver FLT+ cells (52%) and newborn spleen cells (also 52%) (Fig. 3A). In control progeny, the NBS antigen was more prevalent on fetal liver cells (73%) than on newborn spleen cells (39%) (Fig. 3B). BaP treatment did not alter the percent of NBS+ fetal liver cells, but NBS+ cells were significantly increased in newborn spleen (Fig. 3B).

Persistence of transplacental BaP-induced T-cell changes in juvenile progeny

In 3-week-old juveniles, NBS+ cells were still significantly increased in spleens of BaP-exposed progeny, while FLT+ cells were not (Fig. 4A). As the BaP-exposed mice matured, the initial overexpression of FLT and NBS markers was reversed. By 6 weeks, NBS+ cells were significantly depressed in the spleen (Fig. 4B), and FLT+ thymocytes were significantly depressed in BaP-exposed progeny (Fig. 4C), compared with the untreated progeny.

BaP-induced changes in the total number of fetal liver cells expressing Lyt, FLT, or NBS antigens

Changes in cell frequencies resulting from BaP treatment would be influenced by selective toxicity for T-cell subpopulations. The mean total numbers of lymphoid cells recovered from offspring that were either treated or untreated *in utero* with BaP are shown in Table 3. The yield of lymphoid cells per fetal liver showed a 3-fold reduction ($P<0.05$) in BaP-exposed fetuses compared with control fetuses. The results for total numbers of cells/fetal liver expressing antigen markers showed that BaP treatment did not alter the absolute number of FLT+ cells in fetal liver, but did produce a 60% decrease in NBS+ cells in fetal liver. By contrast, Ly1+ cells were reduced by 89%, and Ly2+ cells were reduced by 99%. These results showed that both FLT and NBS antigen expression were relatively resistant to the toxic effects of BaP. The BaP-induced increase in the frequency of FLT+ and NBS+ cells in fetal liver lymphocytes (Fig. 3A and B) is therefore due to the selective decrease in T-cells bearing conventional Lyt differentiation markers.

Table 3. Absolute number of cells/mouse expressing antigens in progeny

Tissue*	Total cell yield ($\times 10^{-7}$)†	Number of cells expressing antigens ($\times 10^{-7}$)‡ ATS	Ly1	Ly2	FLT	NBS
Fetal liver§						
CO-controls	1.41 ± 0.21	0.79	0.46	0.79	0.28	1.03
BaP-treated	0.56 ± 0.26	0.09	0.05	0.006	0.29	0.38
Newborn spleen						
CO-controls	0.77 ± 0.61	0.48	0.50	0.51	0.16	0.18
BaP-treated	0.86 ± 0.14	0.70	0.57	0.44	0.44	0.51
Newborn thymus						
CO-controls	3.85 ± 2.3	2.16	2.31	2.23	1.35	1.54
BaP-treated	2.19 ± 0.21	1.73	1.38	1.66	0.92	0.85
Juvenile spleen						
CO-controls	2.20 ± 0.36	1.23	ND‖	ND‖	0.66	1.21
BaP-treated	2.25 ± 0.26	0.96	ND‖	ND‖	0.38	0.38
Juvenile thymus						
CO-controls	1.49 ± 0.17	1.03	ND‖	ND‖	1.21	0.48
BaP-treated	1.60 ± 0.14	1.26	ND‖	ND‖	0.93	0.45

*Tissues were removed from progeny at 17–19 days gestation (fetal liver), at 3–5 days postpartum (newborn), and at 6 weeks postpartum (juvenile).

†Mean ± S.D. from three experiments.

‡Subpopulation values are calculated from the percentage of cells reactive with antibody (either anti-T-cell antiserum, anti-Ly 1.2 alloantiserum, anti-Ly 2.2 alloantiserum, MAb FLT, or MAb NBS) multiplied by the total cell yield per mouse.

§Values for fetal liver are number of cells per fetus.

‖ND, Not determined.

BaP-induced changes in the total number of spleen and thymus cells expressing Lyt, FLT, or NBS antigens after birth

In newborn and juvenile offspring, no significant differences were demonstrated in the number of total lymphocytes, Ly1, or Ly2 cells recovered from spleens of BaP-treated versus untreated progeny (Table 3). However, total FLT+ and NBS+ cells were increased in newborn spleen, followed by a decrease in 6-week juvenile spleens ($P<0.05$).

In newborn thymus, total thymocytes and all thymocyte subpopulations, including FLT+ and NBS+ cells were depleted in BaP-exposed progeny to 60% or less of normal control levels (Table 3). By 6 weeks of age, total thymocytes and NBS+ thymocytes had recovered to normal control levels, while FLT+ thymocytes remained significantly depressed ($P<0.01$) in BaP-exposed progeny.

DISCUSSION

We have produced monoclonal antibodies that react with putatively novel murine cell surface T-lymphocyte antigens and distinguish significant modulation of T-cell ontogeny by transplacental BaP. Two surface antigens (FLT and NBS) on lymphocytes were identified that showed different cellular distributions in prenatal and postnatal lymphoid tissues. A monoclonal antibody produced to fetal liver T-cells identified a cell subpopulation bearing the FLT marker (FLT+ cells). A monoclonal antibody produced to newborn spleen cells identified a different subpopulation bearing the NBS marker (NBS+ cells). In normal control mice, age-related and organ distribution differences in the frequencies of FLT+ and NBS+ cells suggest that these antigens may be markers of different stages of T-lymphocyte maturation. Thymocytes in juvenile mice were 80% positive for FLT antigen, while thymocytes of mature mice were only 30% positive for FLT. In contrast, the NBS antigen was more prevalent on spleen cells (55–61%) than on thymocytes (30–44%).

The number of cells expressing either antigen was markedly reduced in the thymus and spleen of young mice that had been exposed to BaP *in utero*. Hence, the antigens identified are associated with the toxic effects of BaP for the developing immune system. The exact nature of these surface antigens is not yet known. A T-cell origin is indicated by their presence on purified thymocytes and lymphocyte preparations from neonatal tissues that were shown to contain T-cells but which lack mature (sIgM+) B-cells. We cannot rule out that the antigens are shared with other leukocytes, such as NK (natural killer) cells or pre-B-lymphocytes that could also be present in the perinatal tissues (Owen & Jenkinson, 1981).

BaP-induced changes in the distribution and frequency of the FLT and NBS antigens during ontogeny were studied and compared with changes in the distribution and frequency of Lyt antigens. The effect of transplacental BaP on neonatal Lyt antigens of the BALB/c mouse has not previously been reported. Most studies of BaP toxicity in mice have been carried out in the C3H, C57Bl/6, or C3H × C57Bl/6 F_1 hybrid mouse strains. In genetic studies, these strains have shown maximum induction of aryl hydrocarbon hydrolase which has a role in activation of BaP to the electrophoretic DNA-reactive species, BPDE (Lambert & Nebert, 1977; Shumm *et al.*, 1979). However, BALB/c mice do contain inducible Ah genes (Shumm *et al.*, 1979) and they were chosen for this study in order to achieve the aim of monoclonal antibody production.

Total T-cells and Lyt subsets were drastically depleted in the fetal liver of BALB/c progeny exposed to transplacental BaP. The reduction in Ly2+ cells was greater than the reduction in Ly1+ cells. This finding is in contrast to the results obtained by Johnson & Urso (1989), who observed an increase in Ly2+ cells in the fetal liver and spleen of neonatal C3H mice and increased Ly2+ cells in newborn spleen, following exposure to BaP *in utero*, a condition that persisted into the neonatal period. Despite the difference observed in the response to BaP, the ratio of Ly1 (33%): Ly2 (56%) cells in normal fetal livers of BALB/c mice were similar to those reported (Urso & Johnson, 1987) for prenatal C3H mice (<10% Ly1: 20% Ly2). At 5 days of age, we found a 1:1 ratio of Ly1:Ly2 in the thymus and spleen, which is expected from other observations that peripheral T-cells in the newborn mouse express both markers up to 1 week of age, and begin to differentiate thereafter, with the Ly1+ subset predominating over the Ly2+ subset (Owen & Jenkinson, 1981; Cantor & Boyse, 1975). The ontogeny of the fetal liver T-cells is not known. The excess expression of Ly2+ suggests that they are differentiated cells and might exhibit regulatory functions that are known to be of importance in maintenance of the fetal allograft (Murgita & Wigzell, 1981). Since the mouse thymus is populated by pre-T-cells on about day 11 of gestation, the 19-day fetal liver cells that we have studied may have been derived from extrathymic differentiation or from post-thymic differentiation, followed by repopulation of fetal liver lymphoid tissue.

In contrast to the severe depletions observed in Lyt antigen bearing cells in fetal liver exposed to BaP, the

proportion of cells bearing the NBS antigen was unaffected and remained at 70%, while the FLT+ cells increased from 20% to 50%. This proportional increase could be due either to cellular proliferation or to an innate resistance of these cells to the toxic effects of BaP. Analysis of the data, to account for reduced total numbers of recoverable lymphocytes from BaP-exposed fetal liver, showed that the absolute number of FLT cells in the 19-day fetus remained the same after BaP treatment. Hence, the simplest explanation for the data would be that the antigens identified by both monoclonal antibodies are associated with BaP-resistant cells. An alternative explanation would be that the FLT and NBS antigens are maturational markers that are expressed prior to expression of conventional Lyt antigens. In the latter case, extensive cellular proliferation for repopulation of lymphoid tissues with T-cells, in the prenatal mouse, could have resulted in a disproportionately high percentage of cells expressing FLT and NBS markers without a similar increase in Lyt antigen expression. Following parturition, enhanced frequencies of FLT+ and NBS+ cell types resulting from single-dose prenatal BaP exposures were apparent in the spleen of newborn mice. While Lyt bearing cells were present at normal splenic frequencies, FLT+ and NBS+ cells in newborn spleen from BaP-exposed progeny showed a two-fold increase in frequency over normal controls. This suggests that the FLT+ and NBS+ cells participated in repopulation of the BaP depleted T-lymphocyte pool. In 3-week-old juveniles, NBS+ cells were still significantly enhanced in spleens of BaP-exposed progeny, while FLT+ cells were not. By 6 weeks, the relative frequency of NBS+ cells was significantly depressed in spleen, although the total number of NBS+ cells was similar in BaP-exposed and normal controls (Table 3). In the juvenile thymus, both the relative frequency and the total number of FLT+ thymocytes were significantly depressed in BaP-exposed progeny, compared with corn oil-treated control mice. Although the ultimate decrease in the frequency of expression of FLT and NBS antigens is clearly related to transplacental BaP exposure, the mechanism underlying this phenomenon is unknown. The fluctuations observed suggest that the FLT and NBS antigens are associated with relatively undifferentiated cells (i.e. cells lacking Lyt differentiation markers); hence the decrease could be due to cellular differentiation rather than cell death. In either case, the monoclonal antibodies have identified significant changes in T-cell surface antigens that persist for at least 6 weeks postnatally following prenatal exposure to BaP.

The generation of large numbers of hybridomas producing antibody to the FLT and NBS antigens is a somewhat curious phenomenon. It was initially assumed that a syngeneic immunization with BaP-exposed fetal or neonatal lymphoid cells might detect antigenic changes resulting from the formation of BaP adducts in fetal cell proteins or DNA (Harris *et al.*, 1985; Haugen *et al.*, 1986; Shugart & Malsunami, 1985), which could potentially be exploited as biomarkers of BaP exposure and/or effects. The antibodies produced appear to be highly reactive with normal T-cell surface antigens, and were able merely to detect changes in the frequency and organ distribution of these antigens. Hence, the monoclonal antibodies were produced as the result of a lack of self-tolerance for FLT+ and NBS+ antigens in the mice contributing the spleen cells for hybridization. We have no information on the regulatory mechanisms involved, but it is logical to assume that BaP treatment of progeny that were used to purify cells for immunization made an important contribution to the apparent production of autoreactive antibodies in syngeneic adults. Because BaP caused severe depletions of Lyt+ cells in fetal liver, and enhancement of FLT+ and NBS+ cell proliferation in newborn spleen, a disproportionately large amount of these antigens was present in the lymphocyte preparations from BaP-exposed tissues. Further studies are required to determine the role, if any, of BaP in the production of autoreactive (and alloreactive) anti-T-cell antibodies.

Overall, the results show that FLT and NBS antigen expression on T-cells was highly resistant to the initial toxic effect of BaP for fetal lymphocytes and that FLT+ and NBS+ cells participated in repopulation of lymphoid tissues following prenatal BaP T-cell depletion. It is also apparent that the reduced numbers of these cells in later life, resulting from interaction with BaP during the critical ontogenetic period for T-cell differentiation, could be associated with the depression in cell-mediated immunity and tumor progression that is known to result from *in utero* exposure to this carcinogen.

In conclusion, two MAbs were produced and characterized which identify *in utero* BaP-induced changes in lymphocytes. These findings showed that transplacental BaP alters ontogenic processes in BALB/c lymphocytes from the prenatal to postnatal period, and this animal model suggests the potential use of cellular antigens as biomarkers of chemical exposures that could be associated with human developmental effects.

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