Effects of Prenatal Exposure to Cigarette Smoke on Offspring Tumor Susceptibility and Associated Immune Mechanisms

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Epidemiologic evidence suggests that prenatal exposure to intact (unfractionated) cigarette smoke (CS) increases the incidence of cancer in the offspring. A toxicology study was carried out to examine the effects and underlying mechanisms of prenatal exposure to mainstream cigarette smoke (MCS) on offspring resistance to tumor challenge and surveillance mechanisms critical for the recognition and destruction of tumors. Pregnant B6C3F1 mice were exposed by inhalation to MCS for 5 days/week (4 h/day from gestational day 4 to parturition). Smoke-induced effects on offspring—host resistance to transplanted tumor cells; natural killer (NK) cell and cytotoxic T-lymphocyte (CTL) activity; cytokine levels; lymphoid organ immune cell subpopulations; and histology—were examined in 5-, 10- and 20-week-old male and female offspring. At a concentration of smoke roughly equivalent to smoking <1 pack of cigarettes/day, prenatally exposed male offspring challenged at 5 week of age with EL4 lymphoma cells demonstrated a greater than two-fold increase in tumor incidence (relative to age- and gender-matched air-exposed offspring); tumors in prenatally smoke-exposed pups also grew significantly faster. Cytotoxic T-lymphocyte activity in the smoke-exposed 5- and 10-week-old male pups was significantly less than that of the age- and gender-matched controls. No effects of prenatal CS exposure were observed on offspring NK activity, cytokine levels, lymphoid organ histology, or immune cell subpopulations. Results demonstrated that exposure of pregnant mice to a relevant dose of MCS decreased offspring resistance against transplanted tumor cells and persistently reduced CTL activity in prenatally exposed pups. This study provides biological plausibility for the epidemiologic data indicating that children of mothers who smoke during pregnancy have a greater risk of developing cancer in later life.

Key Words: cigarette smoke; developmental immunotoxicology; tumor surveillance mechanisms.

INTRODUCTION

Despite the well-known adverse health effects of maternal cigarette smoking on the fetus, newborn, and young child, 20–30% of women still smoke cigarettes in their reproductive years (ACOG, 1994). Approximately 1.75 million infants are born each year after in utero exposure to active or passive maternal cigarette smoke (CS) (Byrd and Howard, 1995).


Cigarette smoke contains about 4800 compounds, at least 60 of which are classified as animal and/or human carcinogens (IARC, 2004). Many constituents of CS including nicotine and polycyclic aromatic hydrocarbons (PAH) have been shown to pass the placenta (Perera et al., 2004; Pichini et al., 2000). Cotinine, a metabolite of nicotine with a longer half-life, has been found in cord blood as well as in urine samples of newborns of smoking mothers (Pichini et al., 2000). In a study by Perera et al. (2004), which compared markers of smoke exposure in smoking mothers and their newborns, cotinine levels and benzo[a]pyrene (B[a]P)-DNA adducts were higher in the newborns compared with those measured in blood samples from the mothers.

Although the effect of gestational exposure to CS on childhood cancer risk is still being debated (Sasco and Vainio, 1999), accumulating epidemiologic data indicate that smoking during pregnancy increases the risk of certain cancers (i.e., central nervous system [CNS] tumors, leukemias, and lymphomas) in the prenatally exposed offspring (Filippini et al., 1994, 2000; Magnani et al., 1990; Schuz et al., 2001). For example, a positive association has been observed between...
maternal smoking and CNS tumor incidence in the offspring (Filippini et al., 1994); later epidemiological studies further supported this association (Filippini et al., 2000; Schutz et al., 2001). In another study, maternal smoking was linked to the development of acute non-lymphocytic leukemia and lymphoma (Magnani et al., 1990) during childhood. Support for these population studies comes from a molecular epidemiology study (Finette et al., 1998) which demonstrated that specific genomic deletions, reflecting a recombinational event associated with hematopoietic malignancies in early childhood, were more frequent in newborns exposed to CS in utero than in children from non-smoking mothers.

The immunosuppressive properties of intact (unfractionated) CS, as well as many of its individual constituents, have been well established (McAllister-Sistilli et al., 1998; Sopori and Kozak, 1998). Smoke-induced impairment of the immune system could result in increased susceptibility of the fetus or newborn to the toxic and/or carcinogenic effects of CS. Human studies have demonstrated that long-term direct exposure to CS alters immune parameters by several mechanisms: by increasing circulating white blood cell numbers and percentages of total T-lymphocytes; by suppressing antibody production, CD4/CD8 ratio, natural killer (NK) cell numbers, and activity; and by modulating the number and function of alveolar macrophages (McAllister-Sistilli et al., 1998; Sopori and Kozak, 1998). Studies in rodents have revealed that short-term exposure to mainstream cigarette smoke (MCS) tends to stimulate the immune response, but longer-term exposure produces immunosuppression, including reduced lymphocyte proliferation and decreased resistance to transplanted tumors and/or infectious disease challenge (Johnson et al., 1998).

Although information concerning the effects of maternal smoking on offspring immunocompetence is limited, several studies have confirmed that prenatal exposure to CS can alter neonatal immune parameters. For example, neonatal cord blood from smoking mothers had significantly lower levels of polymorphonuclear leukocytes (Mercelina-Roumans et al., 1996) than those measured in cord blood of newborns from non-smoking mothers. In addition, neonatal cord sera from mothers who smoked contained higher levels of immunoglobulin—IgA, IgM, and IgG—than those measured in offspring of non-smoking mothers (Cederqvist et al., 1984). Toxicological studies have demonstrated similar findings. For example, offspring from pregnant rats treated with nicotine (6 mg/kg per day) on gestational days 4–20 demonstrated suppressed T- and B-lymphocyte responsiveness, which remained sub-responsive to stimulation well into adulthood (Basta et al., 2000).

The current toxicological study sought to determine whether inhalation exposure of pregnant mice to MCS altered susceptibility of the young and/or adult offspring to challenge with transplantable tumor cells. In addition, specific immune surveillance mechanisms critical for the recognition and destruction of developing neoplasms were investigated to provide better understanding of the mechanism(s) by which the observed effects on host susceptibility may have occurred.

MATERIALS AND METHODS

Animals. B6C3F1 male and female mice purchased from Jackson Laboratory (Bar Harbor, ME) were between 9 and 11 weeks of age at the time of arrival. Mice were housed in pairs (females) or individually (males) in polycarbonate cages (with corncob bedding) in temperature-controlled (20–23°C) and humidity-controlled (55% RH) rooms; mice were provided food (Purina 5001 lab chow) and tap water ad libitum. The light/dark cycle was maintained on 12 h intervals. Mice were acclimated for at least 1 week prior to use. All animal procedures were conducted under an animal protocol approved by New York University Institutional Animal Care and Use Committee (IACUC).

Mating and gestation. Animals 10–12 weeks of age and weighing ≥20 g were used for mating. A single male mouse was paired with two females (considered gestational day [GD] 0). After 4 days of pairing, the males were removed, and from GD 4 until parturition the females (two per cage) were exposed to either MCS or filtered air for 4 h/day; 5 days/week (via whole-body inhalation). One to two days prior to giving birth (~GD 18), dams were separated and housed individually. At birth, each mother/offspring set was maintained in clean filtered air, and the number of litters with viable offspring, along with the approximate duration of gestation and litter size, were determined. After weaning (3 week), pups and mothers were separated, the offspring were sexed and the sex ratio was determined; offspring body weight gain was determined from 3–10 weeks of age by daily weighing of pups born on the same day from different litters (7 pups/exposure group).

Experimental design. Groups of 20–28 offspring/sex from randomly selected MCS- and air-exposed control dams (from two different exposures) were used to assess host resistance against tumor challenge. All pups were injected subcutaneously (sc) with EL4 lymphoma cells at 5, 10, or 20 weeks of age; tumor incidence and size, as well as time to tumor formation, were monitored for up to 60 days post-injection. Remaining siblings from the same dams were sacrificed at identical ages and used to assess the effects of prenatal smoke exposure on CTL and NK activities (7–8 pups/sex/exposure regime/age group), histologic profile of the thymus and spleen (3–4 pups/sex/exposure group), lymphoid organ subpopulations (7–8 pups/sex/exposure group), and cytokine production (3–6 pups/sex/exposure group). Histologic profile and immune subsets were also evaluated in 3-week-old mice.

Smoke generation and exposure. Mainstream cigarette smoke was generated from the burning of filtered 1R3F cigarettes (Kentucky Tobacco Research & Development Center, Lexington, KY) using an automated cigarette smoke generation system (Baumgartner-Jaeger CSM 2070, CH Technologies [USA] Inc., Westwood, NJ). Reference cigarettes were stored long-term at 4°C–7°C (55% RH); 24 h prior to use, cigarettes were relocated to a humidor and stored at 20–23°C (55% RH). The continuous smoking machine was adjusted to load and light 4–5 cigarettes simultaneously, each of which produced 2-5 puffs of 35 ml volume/puff under the control of an automatically regulated piston pump that cycled once per minute. Smoke was diluted 90% prior to introduction into the exposure chamber. Filtered dilution air entered the bottom of the generation chamber, and the output was introduced into the top of the exposure chamber. Mice were exposed whole-body to MCS or filtered air in polycarbonate cages with wire mesh tops. Cages were rotated among three racks in the chamber to assure even smoke distribution to all animals. Chamber levels of carbon monoxide (CO) and total suspended particulates (TSP) were monitored throughout the exposure. Particle samples were collected from the exposure chambers every hour (for the entire duration of exposure) on Pallflex Emf filters (Pall Corporation, East Hills, NY); means TSP levels were determined gravimetrically from filters weighed before and after sampling. Chamber CO levels were measured continually over the entire 4-h exposure period using...
a 48C CO Analyzer (Thermo Environmental Instruments Inc, Franklin, MA). Levels of blood cotinine and carboxyhemoglobin (COHb) were determined in separate groups of MCS- and air-exposed dams within 10 min after exposure on GD 19 using a Cotinine MICRO-PLATE EIA kit (OraSure Technologies, Inc., Bethlehem, PA) and an IL 682 Co-Oximeter System (GMI, Inc., Ramsey, MN), respectively (Table 1).

**Tumor challenge study.** EL4 mouse lymphoma cells (ATCC, Manassas, VA), used as the tumor model for these studies, were grown in 75 cm² flasks containing Dulbecco’s modified Eagle’s medium (DMEM; supplemented with 10% horse serum, 1% penicillin-streptomycin, and 1% L-glutamine [Invitrogen Corporation, Carlsbad, CA]); tumor cells were passaged twice before each experiment. Just prior to injection, cells were washed once and resuspended to 5 x 10⁶ cells/ml in phosphate buffered saline (PBS). EL4 cells (at a concentration that yielded a 20–40% tumor incidence [TD20-40] in 5-, 10-, and 20-week-old naive male mice) were injected sc into the right rear thigh of each offspring. The two youngest age groups (i.e., 5- and 10-week-old offspring) were injected with a total of 5 x 10⁵ cells, whereas their 20-week-old counterparts required twice as many cells (1 x 10⁶) to produce an equivalent tumor incidence. Time to tumor formation was determined by daily palpation of the tumor injection site; size of palpable tumors was assessed daily by caliper measurements (Fisher Scientific International, Inc., Hampton, NH). Injected mice were sacrificed at either 60 days post-challenge or when the tumor reached 20 mm in size.

**Cytotoxic T-lymphocyte (CTL) activity.** Ex vivo CTL activity was determined according to a protocol similar to that described by House and Thomas (1995). Briefly, recovered spleen cells, treated with erythrocyte lysing buffer (0.9% [w/v] ammonium chloride; 0.1% [w/v] potassium bicarbonate; 0.03% [w/v] sodium-EDTA) for 3 min (at room temperature), were resuspended to a concentration of 6 x 10⁶ viable cells/ml in Minimum Essential Medium with Earle’s balanced salts (EMEM) (IHR Biosciences, Inc., Lenexa, KS). Soon after, P815 mouse mastocytoma cells (ATCC, Manassas, VA) were treated with mitomycin C (50 g/2–5 x 10⁶ cells), incubated in the dark for 30 min (at 37°C), and resuspended in EMEM to a concentration of 1.2 x 10⁶ viable cells/ml. Splenocytes and cultured P815 cells (each at 0.5 ml) were incubated together (at 37°C in 5% CO₂) at a responder (splenocytes):sensitizer (P815 cells) ratio of 50:1. To assure that splenocytes from air- and CS-exposed offspring had equivalent populations of CD8+ cells at the time the cytotoxic assays were performed, a small aliquot of the total spleen cell suspension was shipped, after 5 days of culture, by overnight delivery to SUNY Upstate Medical University for flow cytometric analyses (see below under Flow Cytometric Analysis). The remaining cells were collected and counted, and the final concentration was set at 5 x 10⁶ viable cells/ml. A second P815 culture (1 x 10⁶ cells), resuspended in 0.5 ml fetal bovine serum (FBS) and incubated with 200 µCi ¹⁵Cr (PerkinElmer Life and Analytic Sciences, Inc., Boston, MA) for 75 min (at 37°C), was resuspended (in EEMEM) to a final concentration of 2 x 10⁶ viable cells/ml. Splenocytes and ¹⁵Cr-labeled P815 cells (each at 0.1 ml) were incubated (at 37°C in 5% CO₂) for 4 h in individual wells of a round-bottom microtiter plate (Corning Incorporated, Corning, NY); separate wells containing ¹⁵Cr-labeled P815 cells were incubated with either EEMEM or 1% Triton-X (Sigma-Aldrich) to determine spontaneous release (SR) and total releasable (TR) counts, respectively. Following incubation, all cells were centrifuged (5 min at 1500 rpm), supernatant (100 µl) from each well removed and radioactive release (in cpm) determined using a γ-scintillation counter (LKB-Wallac: 1275 Minigamma counter, Perkin Elmer Inc., Wellesley, MA). Cytotoxicity was calculated as: % cytotoxicity = ([ER-SR]/[TR-SR]) x 100 where ER, SR, and TR represented experimental release, spontaneous release, and total releasable counts, respectively.

**Natural killer (NK) cell activity.** Natural killer cell activity was determined using a modification of the protocol initially described by Djeu (1995). Briefly, recovered splenocytes were resuspended in RPMI 1640 media (40 ml) and incubated for 1 h at 37°C. Non-attached cells were recovered and resuspended in RPMI to a final concentration of 2 x 10⁶ viable cells/ml. At the same time, 5 x 10⁶ cultured YAC1 mouse lymphoma cells (ATCC) were incubated (at 37°C for 1 h) with 200 µCi ¹⁵Cr and then diluted (in RPMI) to a final concentration of 1 x 10⁶ viable cells/ml. Splenocytes and ¹⁵Cr-labeled YAC1 cells (each at 0.1 ml) were then incubated (at 37°C in 5% CO₂) for 4 h in individual wells of a round-bottom microtiter plate and cytotoxicity calculated in the same manner as that described for CTL activity.

**Flow cytometric analysis.** Freshly isolated spleen and thymus cells, treated with erythrocyte lysing buffer for 5 min (at room temperature), were kept in ice-cold Minimum Essential Medium with Hank’s salts (HEM; supplemented with 15% FBS, 1% penicillin-streptomycin, and 1% L-glutamine [Invitrogen Corporation]) and shipped by overnight delivery to SUNY Upstate Medical University for flow cytometric analyses. Before staining, spleen cells were first Fc blocked using purified rat anti-mouse CD16/32 (clone 2.4G2, BD Pharmingen, San Diego, CA). Aliquots of 10⁶ cells were stained in 40 µl of 0.5% BSA/0.1% NaN₃/PBS with previously titrated amounts of fluorochrome-conjugated or biotinylated antibodies for 15 min (at 4°C) in the dark, washed, and then reincubated with streptavidin-PerCP for 15 min if needed. After staining, cells were washed once and resuspended in 500 µl of 0.5% BSA/0.1% NaN₃/PBS and analyzed by flow cytometry within 2 h. Cells were stained with various six color combinations (Table 2); isotype controls for staining were performed with the same cells from each mouse (all antibodies and SA-PerCP were purchased from BD Pharmingen). Data from 30,000–100,000 cells from each sample were collected using the BD FACSDiva software on a LSR-II flow cytometer (Becton Dickinson, San Jose, CA) with an Octagon 488 nm blue laser and a Red Trigon 633 nm red laser. Dead cells and debris were excluded by gating on forward scatter versus 90° scatter for analysis. Quantification of selected populations was done using the BD FACSDiva software.

**Cytokine measurements.** Levels of tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-1β, and IL-4 in the lavage fluid and serum of the air- and smoke-exposed offspring were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Biosource International, Camarillo, CA).

**Histopathology.** The spleen and thymus of prenatally exposed offspring were evaluated histologically. The tissues were fixed in 10% neutral buffered formalin and subsequently processed to—and embedded in—paraffin wax (Fisher Scientific). Sections (5 µm-thick) were prepared and stained with hematoxylin and eosin (Richard-Allan Scientific, Kalamazoo, MI).

**Statistical analysis.** The means of 2–3 replicate values for each ex vivo assay (for a given sample from a single mouse) were calculated, and the resulting values were used for statistical analyses. All experiments were performed in triplicate and analyzed using one-way ANOVA (α = 0.05). Significant differences were determined using Tukey’s honestly significant difference post-hoc test. All data were analyzed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA).
performed twice (with one set of animals from each exposure) for a given end point, and data from the two experiments were pooled. Survival analysis for the tumor challenge studies was performed by GraphPad Prism 4.0a (GraphPad Software, Inc., San Diego, CA). One-way analysis of variance (ANOVA) was used to determine CS-induced effects on tumor growth, CTL activity, NK cell cytotoxicity, lymphoid subpopulation profiles, and cytokine production. Fisher’s Protected LSD analysis was used for post-hoc testing when appropriate (Abacus Concepts, Inc., Berkeley, CA). Differences were considered significant when probability ($p$) values were $<0.05$.

RESULTS

Gestational Parameters

Although litter number was similar between exposure groups, prenatal exposure to CS significantly reduced litter size by 11% (compared to offspring exposed prenatally to filtered-air) (Table 3). In addition, CS exposure in utero shifted offspring sex ratio; 23% fewer male pups were born to CS-exposed dams than to their non-smoking counterparts. Although mice were paired for 4 days and, thus, gestational length could only be defined ± 4 days of uncertainty, prenatal CS exposure seemed to have had no effect on duration of gestation. Offspring body weight (BW) gain was unaltered by prenatal CS exposure. Male and female offspring from smoke-exposed dams gained 347 (± 42 [SE]) mg/day and 245 (± 26 [SE]) mg/day from 3 to 10 weeks of age, respectively, whereas gender-matched control counterparts gained 358 (± 45 [SE]) mg/day and 242 (± 31 [SE]) mg/day, respectively.

Tumor Incidence and Growth

Prenatal exposure to CS altered EL4-induced tumor incidence in challenged offspring (Fig. 1A). Five-week-old male pups from smoke-exposed mothers demonstrated a 2.3-fold increase in tumor growth compared to sex- and age-matched air-exposed offspring; a similar trend was observed for the 5-week-old female offspring ($p = 0.07$) and the 10-week-old male offspring ($p = 0.09$). Tumor incidence of 10-week-old female pups and 20-week-old offspring of either sex (compared to age-/gender-matched air control mice) was unaffected by prenatal smoke exposure. Interestingly, differences in tumor incidence between the sexes were also influenced by host age. Despite the administration of identical doses of tumor cells, air- and smoke-exposed 20-week-old male offspring demonstrated a significantly higher tumor incidence than their age-matched female counterparts.

EL4-induced palpable tumors grew significantly faster in the 5-week-old male offspring exposed prenatally to CS than such tumors in the air-exposed pups or their age-matched female counterparts (Fig. 1B). Tumor growth rate in the 10-week-old male offspring exposed to smoke in utero followed a similar trend ($p < 0.1$).

Prenatal exposure to CS had no effect on time to tumor formation in any age group of either sex (Fig. 1C). Tumors in

TABLE 2

Six Color Combinations of Immunofluorescence Staining

<table>
<thead>
<tr>
<th>Stains</th>
<th>CD8 (53–6.7)</th>
<th>B220 (RA3-6B2)</th>
<th>CD45RB (16A)</th>
<th>CD11b/Mac-1 (M170)</th>
</tr>
</thead>
<tbody>
<tr>
<td>488-nm blue laser</td>
<td>FITC</td>
<td>PE</td>
<td>Biotin+ SA-PerCP</td>
<td>FITC</td>
</tr>
<tr>
<td></td>
<td>CD25 (7D4)</td>
<td>CD25 (7D4)</td>
<td>CD25 (7D4)</td>
<td>CD25 (7D4)</td>
</tr>
<tr>
<td></td>
<td>PE-Cy7</td>
<td>CD3 (145–2C11)</td>
<td>CD3 (145–2C11)</td>
<td>CD22 (12–2C11)</td>
</tr>
<tr>
<td></td>
<td>PE-70</td>
<td>CD4 (GK1.5)</td>
<td>CD4 (GK1.5)</td>
<td>CD4 (GK1.5)</td>
</tr>
<tr>
<td></td>
<td>PerCP</td>
<td>Biotin+ SA-PerCP</td>
<td>CD11b/Mac-1 (M170)</td>
<td>FITC</td>
</tr>
<tr>
<td></td>
<td>CD44 (1M7)</td>
<td>CD4 (GK1.5)</td>
<td>CD4 (GK1.5)</td>
<td>CD4 (GK1.5)</td>
</tr>
</tbody>
</table>

TABLE 3

Effects of In Utero Cigarette Smoke Exposure on Gestational Parameters

<table>
<thead>
<tr>
<th>Gestational parameters</th>
<th>Air</th>
<th>Cigarette smoke</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration of gestation (d)$^{a}$</td>
<td>20.9 ± 0.2</td>
<td>21.2 ± 0.2</td>
</tr>
<tr>
<td>Litter size (no. of viable pups/litter)</td>
<td>8.7 ± 0.2</td>
<td>7.7 ± 0.2$^{*}$</td>
</tr>
<tr>
<td>No. of litter with viable offspring</td>
<td>79 (104)$^{c}$</td>
<td>73 (104)$^{c}$</td>
</tr>
<tr>
<td>Female / male ratio</td>
<td>1.0 ± 0.04</td>
<td>1.3 ± 0.09$^{*}$</td>
</tr>
</tbody>
</table>

$^{a}$Values represent the mean ($n = 79$ and 73 litters in the air- and smoke-exposed groups, respectively) ± SE.

$^{b}$Based on the first day of pairing (considered for this study as gestational day 0); mice were paired for 4 days.

$^{c}$Total number of paired females.

$^{*}$Significantly different ($p < 0.05$) from the air-exposed control.
offspring of both sexes from the two exposure regimes were palpable 19 and 21 days post-injection for the 5- and 10-week-old tumor-challenged offspring, respectively. Even though statistical analyses could not be performed for the 20-week-old female offspring (because of inadequate sample size), tumor development in the oldest age group (from both exposure regimes of the two sexes) appeared delayed, with masses only becoming palpable at about 30 days post-injection.

**CTL Activity**

Effect of MCS on CTL activity was sex-dependent, with the males proving more sensitive to the effects of prenatal smoke exposure than their age-matched female counterparts (Fig. 2). Exposure in utero to CS significantly reduced CTL activity in the 5- and 10-week-old male offspring compared to the age- and sex-matched air-exposed control groups. In contrast, CTL activity in the female pups (of any age) was unaffected by prenatal MCS exposure. Host age also influenced CTL activity, particularly in the case of the female offspring. Cytotoxic T-lymphocyte activity decreased significantly with advancing age in the female offspring from nonsmoking mothers. In contrast, activity measured in the air-exposed male offspring decreased more slowly, reaching statistical significance (compared to their 5-week-old counterparts) only in the 20-week-old age group.

**NK Cytotoxicity**

Natural killer cell activity for either sex of any age group was unaffected by prenatal CS exposure. However, NK activity differed significantly between the sexes (Fig. 3). Five- and 10-week-old air-exposed male offspring demonstrated significantly lower NK activity than their age-matched female counterparts. In a pattern similar to that observed for CTL activity, NK activity decreased significantly with increasing age in the air-exposed female offspring.

**FIG. 1.** Effects of prenatal cigarette smoke (CS) exposure on tumor incidence (A), tumor growth rate (B), and time to tumor formation (C) after sc injection of 5-, 10-, and 20-week-old offspring with cultured EL4 lymphoma cells. Tumor incidence is defined as the percentage of prenatally exposed offspring with palpable tumors \( n = 28 \) EL4-injected pups/sex/exposure group for 5- and 10-week-old mice; \( n = 20–21 \) EL4-injected pups/sex/exposure group for 20-week-old mice). Tumor growth rate reflects the mean value (± SE) of the daily increase in palpable tumor size (value in parentheses indicates the number of pups with palpable tumors after EL4 injection). Time to tumor formation is defined as the number of days (± SE) after injection that a tumor first became palpable (value in parentheses indicates the number of pups with palpable tumors). *Significantly different \((p < 0.05)\) from the sex- and age-matched air-exposed offspring. aSignificantly different \((p < 0.05)\) from the age- and exposure-matched female counterparts.

**FIG. 2.** Effects of prenatal cigarette smoke (CS) exposure on offspring cytotoxic T-lymphocyte (CTL) activity. Values represent the mean \((n = 7–8\) mice/sex/exposure regime/age group) ± SE. *Significantly different \((p < 0.05)\) from the sex- and age-matched air-exposed offspring. *Significantly different \((p < 0.05)\) from the age- and exposure-matched female counterparts. *Significantly different \((p < 0.05)\) from the sex- and exposure-matched 5-week-old offspring. *Significantly different \((p < 0.05)\) from the sex- and exposure-matched 10-week-old offspring.
Lymphoid Organ Immune Cell Subpopulations

CD8\(^+\) cells (for use in the CTL assay) were evaluated by flow cytometry from cultured splenocytes and were found to be equally distributed among splenic populations from both the air-exposed and CS-exposed offspring (data not shown). Effects of prenatal exposure to CS on spleen and thymus subpopulations in the different age groups of offspring were examined by flow cytometry, and no differences between the smoke-exposed and air-exposed groups were observed for any of the cell phenotypes examined in this study (Table 2). Data from the 5-week-old age group are presented as an example of these findings. As shown in Figures 4A and 4B, respectively, prenatal exposure to CS had no effect on any percentage of immune cell phenotype examined in the spleen (i.e., B-lymphocytes, CD4\(^+\) T-cells, CD8\(^+\) T-cells, NK cells, or granulocytes measured within the lymphocyte gate) or thymus (CD4\(^-\)CD8\(^+\), CD4\(^+\)CD8\(^-\), CD4\(^-\)CD8\(^-\), and CD4\(^+\)CD8\(^-\)) for offspring of any age group. The four subsets (TN1 to TN4) of the thymocyte double-negative population (CD4\(^-\)CD8\(^-\)) and T-regulatory cells (CD4\(^+\)CD25\(^+\)) were also unaffected by prenatal CS exposure (data not shown). In addition, the CD4/CD8 ratio for mature thymocytes in smoke-exposed 5-week-old offspring was similar to that seen for the age-matched male (2.5:1) and female (2.7:1) air-exposed controls.

Cytokine Production

Effects of prenatal exposure to CS on bronchopulmonary lavage and serum cytokine levels were determined in 5-, 10-, and 20-week-old offspring, and lavage data for the 5-week-old age group are shown (as an example) in Figure 5. Levels of TNF-\(\alpha\), IL-1\(\beta\), and IL-4 were unaffected by prenatal exposure to CS in either sex of any age. Effects of CS on IFN-\(\gamma\) could not be accurately assessed as the levels were below the detectable range of the assay (\(\leq 7.8\) pg/ml).

Histopathological Evaluation

There were no qualitative differences in the histologic appearance of the spleen or thymus between the offspring exposed prenatally to CS or filtered air. Specifically, there were no apparent differences in the thickness of the cortex, ratio of cortex to medulla, or cellular composition of cortex and medulla in the thymus. For the spleen, no apparent differences were observed in the ratio of red to white pulp, cellular composition of red pulp and white pulp, or extent of hematopoietic activity in the spleen. Overall, no CS exposure–related lesions were observed in either lymphoid tissue.

DISCUSSION

Results of this study demonstrated that exposure of pregnant mice to a low concentration of MCS (related to smoking <1 pack of cigarettes per day) from GD 4 to parturition increased the incidence and growth rate of EL4-induced tumors in the
offspring of smoking mothers. These findings confirm previous toxicologic studies demonstrating that exposure to CS or its individual constituents can modulate tumor development in challenged hosts (Chalmer et al., 1975; Urso and Gengozian, 1984). For example, transplanted B16 melanoma cells administered sc grew faster in adult male C57BL/6J mice exposed (via inhalation) to a mixture of CS and air (1:7 CS to air ratio [7-8 min/day] for varying time periods up to 30 week) than in their sex- and age-matched controls (Chalmer et al., 1975). Mice also demonstrated significantly more lung metastases than their air-exposed counterparts. In a study examining the effects of a single CS constituent on tumor development in prenatally exposed offspring, Urso and Gengozian (1984) demonstrated that adult C3H/Anf mice exposed in utero to B[a]P (150 μg/g BW of dams) from GD 11–17 had an 8- to 10-fold higher incidence of tumors than those exposed prenatally to the vehicle control. Results from the aforementioned studies, together with the findings from the present study, indicate that both direct and prenatal exposure to CS and/or its constituents can increase host susceptibility to tumor growth and development.

Although the particular smoke constituent(s) responsible for the observed effects on offspring tumor incidence and/or growth is not known, many immunomodulatory (Sopori, 2002) and genotoxic (DeMarini, 2004) CS constituents (including PAHs, nicotine, and N-nitrosamine compounds) can cross the placenta (Perera et al., 2004; Pichini et al., 2000) and, potentially, exert effects on the fetal immune system. A number of previous studies have demonstrated the immunomodulatory effects of unfractionated CS and/or its individual constituents on host immune mechanisms important for tumor surveillance. For example, short-term in vitro exposure to MCS condensates suppressed female C57BL/6 mouse peritoneal macrophage functional capabilities (i.e., phagocytosis of Ig-opsonized sheep red blood cells, H2O2 production, class II major histocompatibility complex expression, and nitric oxide synthesis) induced by IFN-γ (Braun et al., 1998). In a study by Kalra et al. (2004), Lewis rats treated with nicotine-containing patches for 3-4 weeks demonstrated suppressed mitogen-induced T-cell proliferation and mobilization of intracellular Ca2+ by spleen cells. In vivo exposure to another CS constituent, 7,12-dimethylbenz[a]anthracene (DMBA) suppressed mitogen- and alloantigen-induced lymphoproliferation, CTL cytotoxicity, and NK cell cytolysis in B6C3F1 female mice after repeated administration of DMBA over 2 weeks (Ward et al., 1986). Thus, CS-induced effects on immune tumor surveillance mechanisms likely play an important role in the enhanced host susceptibility to tumor development and growth observed in this study.

The present study demonstrated that prenatal exposure to CS reduced CTL activity in the male offspring (compared to age- and gender-matched air-exposed offspring), and that this effect persisted for up to 10 weeks after birth. A number of toxicological studies have previously demonstrated a relationship between CS-enhanced host susceptibility to tumor growth and/or development and suppressed T-lymphocyte function. In an early study by Chalmer et al. (1975), increased growth rates of B16-induced lymphomas in CS-exposed mice were associated with a depression in lymphocyte-mediated tumor-specific cytotoxic responses. Given that CTLs are major effector cells critical for tumor cell lysis (Anichini and Mortarini, 2002), it could be speculated that the increased susceptibility of the male offspring to EL4-induced tumors in this study were due, at least in part, to the observed suppression in CTL activity. This is supported by a number of studies demonstrating the importance of CTL activity in mediating EL4-induced tumor cell growth and development. Maccubin et al. (1989) demonstrated that anti-EL4 CTL responses in C57BL/6 mice exhibited bell-shaped kinetics, with the response correlating with the mean survival time of the EL4 tumor-bearing hosts. In addition, Ho et al. (1993) demonstrated in C57BL/6 mice that the effectiveness of a combined chemotherapeutic treatment against an EL4-induced lymphoma was dependent on the presence of functional CD8+ cells. In that study, long-term survival was completely ablated by pretreatment of mice with anti-CD8 monoclonal antibody (mAb); prior treatment of mice with anti-CD4 mAb only partially inhibited the therapeutic effects, and anti-NK1.1 mAb had no effect.

The observed lack of CS-induced effects on thymus and spleen cell subpopulations and lymphoid organ histology in the prenatally exposed offspring in the present study suggests that reduced CTL activity was associated with a functional defect rather than a reduction in immune cell numbers. That starting populations of splenic CD8+ cells in both exposure groups were equivalent supports this notion. Furthermore, the fact that equal numbers of CD8+ cells were generated after 5 days of culture suggests that, although these cells could proliferate in response to specific challenge, the CTL function was still reduced. Although the mechanism(s) by which CS altered CTL activity in the smoke-exposed offspring is not known, a number of alternatives could be proposed, including T-cell...
anergy (Sopori and Kozak, 1998), mutational defects (DeMarini, 2004), and/or an increase in T-regulatory cells that suppress CTL activity (Shimizu et al., 1999). Sub-chronic and chronic exposure of rats to CS or nicotine alone have been shown to decrease the activation of protein tyrosine kinase (PTK) and phospholipase C-gamma1 (PLC\gamma1) activities; inositol 1,4,5-trisphosphate (IP\text{3})-sensitive Ca\textsuperscript{2+} stores; ability to raise intracellular Ca\textsuperscript{2+} levels; and, lymphoproliferative response to T-cell antigen receptor ligation (Kalra et al., 2000; Sopori and Kozak, 1998). Failure to respond to an antigen, through defective T-cell activation, could account for—or contribute to—the suppressed CTL activity exhibited by the male offspring exposed prenatally to CS.

While T-cell anergy provides one possible explanation by which CTL activity in the offspring may have been suppressed by prenatal CS exposure, a second possibility is related to genotoxic impact on the developing immune system (DeMarini, 2004; Finette et al., 1998; Rodriguez et al., 2002). Human studies have demonstrated that many CS constituents are genotoxic as higher frequencies of micronuclei, sister chromatid exchanges, chromosomal aberrations, chromosome instability, and DNA strand breaks have been found in peripheral lymphocytes of smokers (DeMarini, 2004). Genotoxic CS constituents, which are able to cross the placenta, could exert their effects on developing fetal lymphocytes, leading, ultimately, to T-lymphocyte dysfunction. Moreover, Rodriguez et al. (2002) demonstrated that ip injection of pregnant C3H/HeB mice with B[a]P (150 \mu g/g BW of dams) at GD 11 resulted in B[a]P-DNA adducts in fetal and neonatal T lymphocytes. Authors of the latter study speculated that the early molecular alterations in immature T cells could interfere with normal development and cause altered T-cell functions, leading to immunodeficiency later in life and, ultimately, a more vulnerable environment for tumor induction and growth. Regulatory T cells have been shown to suppress CTL activity (Shimizu et al., 1999), but no significant difference in percentages of CD4\textsuperscript{+}CD25\textsuperscript{+} T cells from the spleens of animals prenatally exposed to CS (compared to controls) was detected in this study. Therefore, it is believed that either anergy induction or mutational defects in CD8\textsuperscript{+} cells caused by the direct action of CS or nicotine are the main possibilities for the observed reductions in tumor immunity.

Cigarette smoke exposure in utero shifted offspring sex ratio, with fewer male pups born to CS-exposed dams. Alterations in offspring sex ratio after prenatal CS exposure have also been observed in epidemiologic studies. For example, Fukuda et al. (2002) demonstrated that offspring sex ratio (numbers of female to male offspring) was skewed when either one or both parents smoked more than 1 pack of cigarettes per day (compared with families in which neither parent smoked). This same type of response has also been observed after exposure to other environmental contaminants, such as dioxins. Ryan et al. (2002) demonstrated that paternal exposure (prior to conception and at the time of conception) to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and other dioxins was associated with the birth of more female children. Ikeda et al. (2005) have also shown that the sex ratio (percentage of male pups) of rat pups (F\textsubscript{2}) was reduced in TCDD-exposed animals compared with controls.

Effects of prenatal CS exposure on tumor development and CTL activity were most dramatic in the male offspring. Differential sensitivity between the sexes in response to prenatal CS exposure (or to its individual constituents) has also been observed in a number of other toxicological studies. For example, chronic oral exposure of female C57BL/6 mice to 200 \mu g/ml nicotine (free base in 2% saccharin) for 30 days prior to—and during—pregnancy caused persistent sex-dependent changes in offspring behavior. Male, but not female offspring, exhibited significant locomotor hyperactivity for up to 2 months after birth (Pauly et al., 2004). Moreover, in a study investigating the effects of prenatal exposure to environmental tobacco smoke (ETS) on adult cardiovascular disease, Yang et al. (2004) demonstrated that atherosclerotic lesion formation, mitochondrial DNA damage, antioxidant activity, and oxidant load in the cardiovascular tissue of adult apolipoprotein E\textsuperscript{−/−} mice were altered most dramatically in the male offspring. Taken together, these studies demonstrate an increased sensitivity of males (compared to females) to the toxic effects of prenatal CS exposure. Enhanced vulnerability of the male has also been observed in response to other toxic pollutants such as benzene (Corti and Snyder, 1998). Such sex differences could have a hormonal basis; however, a causal relationship for these toxicologic observations remains to be elucidated.

In conclusion, this study has established biological plausibility for the epidemiologic data (Filippini et al., 1994, 2000; Magnani et al., 1990; Schuz et al., 2001) indicating that offspring of mothers who smoke may be at greater risk for developing cancer later in life. Although more studies utilizing additional tumor cell models, as well as long-term rodent carcinogenicity studies are needed, it appears that prenatal exposure to MCS increases susceptibility of the offspring (particularly the males) to induced tumor development well after birth. While the exact mechanisms underlying this response are unclear, CS-induced effects on CTL activity appear a likely target.

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