Effects of Chloro-*s*-Triazine Herbicides and Metabolites on Aromatase Activity in Various Human Cell Lines and on Vitellogenin Production in Male Carp Hepatocytes

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We investigated a potential mechanism for the estrogenic properties of three chloro-*s*-triazine herbicides and six metabolites *in vitro* in several cell systems. We determined effects on human aromatase (CYP19), the enzyme that converts androgens to estrogens, in H295R (adrenocortical carcinoma), JEG-3 (placental choriocarcinoma), and MCF-7 (breast cancer) cells; we determined effects on estrogen receptor-mediated induction of vitellogenin in primary hepatocyte cultures of adult male carp (*Cyprinus carpio*). In addition to atrazine, simazine, and propazine, two metabolites—atrazine-desethyl and atrazine-desisopropyl—induced aromatase activity in H295R cells concentration-dependently (0.3–30 μM) and with potencies similar to those of the parent triazines. After a 24-hr exposure to 30 μM of the triazines, an apparent maximum induction of about 2- to 2.5-fold was achieved. The induction responses were confirmed by similar increases in CYP19 mRNA levels, determined by reverse-transcriptase polymerase chain reaction. In JEG-3 cells, where basal aromatase expression is about 15-fold greater than in H295R cells, the induction responses were similar but less pronounced; aromatase expression in MCF-7 cells was neither detectable nor inducible under our culture conditions. The fully dealkylated metabolite atrazine-desethyl-desisopropyl and the three hydroxylated metabolites (2-OH-atrazine-desethyl, -desisopropyl, and -desethyl-desisopropyl) did not induce aromatase activity. None of the triazine herbicides nor their metabolites induced vitellogenin production in male carp hepatocytes; nor did they antagonize the induction of vitellogenin by 100 nM (EC_{50}) 17β-estradiol. These findings together with other reports indicate that the estrogenic effects associated with the triazine herbicides *in vivo* are not estrogen receptor-mediated, but may be explained partly by their ability to induce aromatase *in vitro*. *Key words:* antiestrogenic, aromatase, atrazine, carp, chloro-*s*-triazines, CYP19, estrogenic, H295R, hepatocytes, herbicides, JEG-3, MCF-7, vitellogenin. *Environ Health Perspect* 109:1027–1031 (2001). [Online 26 September 2001]
http://ehpnet1.niehs.nih.gov/docs/2001/109p1027-1031sanderson/abstract.html

The 2-chloro-*s*-triazine family of herbicides, widely used to control broad-leaved and grassy weeds, includes the chemicals atrazine, simazine, and propazine. Triazine herbicides have been used increasingly since the 1960s, particularly on maize crops, in North America and Europe. The estimated use of atrazine alone in the United States was almost 35,000 tons in 1993 (*1*). As a result it is found in relatively high concentrations in surface waters in certain parts of the North American continent (*2*). Triazine herbicides are relatively persistent to abiotic and biotic breakdown (*2,3*) producing detectable levels in drinking water, foods, and fish (*2*).

Epidemiologic studies have associated long-term exposures to triazine herbicides with increased risk of ovarian cancer in female farm workers in Italy (*4*) and of breast cancer in the general population of Kentucky in the United States (*5*). In experiments with female F344 rats, atrazine induced tumors of the mammary gland and reproductive organs (*6*). In female Sprague-Dawley rats, atrazine caused lengthening of estrous cycle and a dose–dependent increase in plasma levels of 17β-estradiol (*7*). Atrazine also caused an earlier onset of the incidence of mammary and pituitary tumors in this rat strain (*7*), a response typical of exposure to exogenously administered estrogens (*8,9*). Recently, atrazine exposure during lactation has been shown to suppress suckling-induced prolactin release in female Wistar rats (*10*). Further, the lactationally exposed male offspring of the atrazine-exposed dams had an increased incidence of prostatitis (*10*), an effect also induced by exposure to exogenous 17β-estradiol (*11*). A subsequent study in Long-Evans and Sprague-Dawley rats has attributed the effects of atrazine on serum prolactin levels to alterations in the hypothalamic control of the release of this hormone by the pituitary (*12*).

Investigations into the mechanism of these apparent estrogenic effects have not been able to demonstrate any consistent interactions of triazine herbicides with the estrogen receptor or effects on receptor-mediated responses (*13–15*). Effects on enzymes involved in steroid synthesis or metabolism have been limited to a study of the inhibition of testosterone metabolism in the anterior pituitary of rats exposed *in vivo* or of whole anterior pituitaries exposed *in vitro* to atrazine (*16*). Weak inhibitory effects were observed on testosterone 5α-reductase (20–37%) at an atrazine concentration of 0.5 mM; a similar observation was made for the deethylated metabolite atrazine-desethyl (*16*). Taken together, effects of atrazine and other triazine herbicides on estrogen receptor function or enzymes involved in sex hormone metabolism have been inconsistent and occurred at extremely high concentrations.

Triazine herbicides are known to be metabolized in various mammals (*17–19*) and chickens (*3*). In human liver microsomes, the major metabolites formed are the mono-dealkylated forms of atrazine: atrazine-desethyl and atrazine-desisopropyl; hydroxylation of the isopropyl groups present in atrazine and propazine also occurs, but to a lesser extent (for structures see Figure 1). Other metabolites formed *in vivo* and found in human urine are the fully dealkylated metabolite of the triazines (atrazine-desethyl-desisopropyl) and several 2-hydroxylated metabolites. Triazine metabolism is catalyzed primarily by cytochrome P450 (CYP) enzymes (*19*). The fully dealkylated metabolite of atrazine, like atrazine, has been shown to have little interaction with the estrogen receptor (*14*). Other than this, little or no toxicologic information is available for the metabolites of triazine herbicides.

Recently, we reported the ability of atrazine, simazine, and propazine to induce aromatase activity in a human adrenocortical carcinoma cell line (*20*). This response was observed at concentrations in the submicromolar range. In the present study we have continued to examine the effects of triazine herbicides and several of their common

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We thank B. Defize of the Hubrecht Laboratory, Utrecht, for the use of their FluorImager. We thank S. Laws at the National Health and Environmental Effects Research Laboratory, U.S. EPA, for helpful discussions.

Received 13 November 2000; accepted 4 April 2001.

metabolites on aromatase activity in several human cell lines—the H295R adrenocortical, JEG-3 placental, and MCF-7 breast cancer cell line. The rationale for choosing JEG-3 cells was to examine the inducibility of aromatase in a system where the enzyme is known to be expressed at relatively high levels compared to the H295R cells; we chose MCF-7 cells to test whether the triazines could induce aromatase activity in a system where the enzyme is normally expressed at very low levels. In addition, we have examined the effects of the triazines and their metabolites on estrogen receptor-mediated vitellogenin expression in cultured primary hepatocytes of male carp (*21*). Increased synthesis of vitellogenin, a yolk-precursor protein in fish and birds, is a response highly sensitive to estrogens and also occurs after exposure to other compounds that are agonists for the estrogen receptor.

Materials and Methods

Cell culture conditions. We obtained H295R, JEG-3, and MCF-7 cells from the American Type Culture Collection (ATCC No. CRL-2128, HTB-36, and HTB-22, respectively). H295R cells were grown in 1:1 (v/v) Dulbecco's modified Eagle medium/Ham's F-12 nutrient mix (DMEM/F12; GibcoBRL, Breda, The Netherlands) containing 365 mg/mL L-glutamine and 15 mM HEPES (GibcoBRL). The mix was further supplemented with 10 mg/L insulin, 6.7 µg/L sodium selenite, and 5.5 mg/L transferrin (ITS-G; GibcoBRL), 1.25 mg/L bovine serum albumin (Sigma, St. Louis, MO, USA), 100 U/L penicillin/100 µg/L streptomycin (GibcoBRL) and 2% steroid-free replacement serum Ultroser SF (Soprachem, France). JEG-3 cells and MCF-7 cells were cultured in DMEM containing 4,500 mg/L D-glucose and 110 mg/L sodium pyruvate (GibcoBRL), 10% heat-inactivated fetal calf serum (ICN, Costa Mesa, CA, USA), and 100 U/L penicillin/100 µg/L streptomycin (GibcoBRL). MCF-7 cells were cultured in DMEM supplemented with L-glutamine, 4,500 mg/L D-glucose, and sodium pyruvate (GibcoBRL) For the aromatase experiments, cells were treated as described previously (*20*). In brief, cells (about 1–2 × 10^5 cells/well) in 24-well culture plates containing 1 mL medium per well were exposed to various concentrations (0, 0.3, 1.0, 3.0, 10.0, and 30 µM) of the triazine herbicides or their metabolites (Riedel-deHaen, Seelze, Germany) (see structures in Figure 1) dissolved in 1 µL of dimethyl sulfoxide (DMSO; Sigma). Negative control cells received 1 µL of DMSO. Positive control cells were exposed to 100 µM of 8-bromo-cyclic adenosine monophosphate (8Br-cAMP) dissolved in medium containing 0.1% DMSO. We included unexposed cells as further controls, and we tested all treatments in quadruplicate.

For the reverse-transcriptase polymerase chain reaction (RT-PCR) experiments, we exposed cells in 12-well plates to 2 µL DMSO or the test chemicals in DMSO; a positive control (100 µM 8Br-cAMP) was included on each plate. We tested each treatment in triplicate and reproduced each experiment three times. DMSO at 0.1% had no effect on CYP19 expression or catalytic activity relative to unexposed cells. The test chemicals did not cause cytotoxicity at concentrations of 30 µM and below, based on visual inspection of the cells, cell attachment, protein content of the wells, and the inability of the triazines to decrease the mitochondrial activity of succinate dehydrogenase determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test (*22*). We determined protein concentrations by the fluorometric method of Udenfriend et al. (*23*), using bovine serum albumin (Sigma) as standard. We added triazines to the cell culture medium at concentrations below their aqueous solubility limit [e.g., 300 µM for atrazine; 50 µM for simazine (*24*)]. All exposures were for 24 hr.

Isolation and amplification of RNA. We isolated RNA using the RNA Insta-Pure System (Eurogentec, Liège, Belgium) according to the enclosed instructions and stored it at –70°C. We performed RT-PCRs using the Access RT-PCR System (Promega, Madison, WI, USA) with various modifications reported previously (*20*). We verified the purity of the RNA preparations by denaturing agarose gel electrophoresis. We obtained suitable primer pairs by entering the human CYP19 cDNA sequence obtained from the European Molecular Biology Laboratories database (Heidelberg, Germany) into the software program Geneworks (version 2.4; IntelliGenetics, Mountain View, CA, USA). The primer pair used for CYP19 mRNA amplification was 5′-TTA-TGA-GAG-CAT-GCG-GTA-CC-3′ and 5′-CTT-GCA-ATG-TCT-TCA-CGT-GG-3′, producing an amplification product of 314 base pairs. As reference, RT-PCR was performed on β-actin mRNA using the primer pair 5′-AAA-CTA-CCT-TCA-ACT-CCA-TC-3′ and 5′-ATG-ATC-TTG-ATC-TTC-ATT-GT-3′, according to the instructions of the Access RT-PCR kit, except using 1 mM $MgSO_4$, an annealing temperature of 54°C, and 25 cycles. We found β-actin mRNA unaffected by any of the treatments (DMSO, triazines, metabolites or 8Br-cAMP) and could be used reliably as a reference amplification response. Detailed information on PCR conditions and reproducibility and ability of the method to be used (semi)quantitatively was published previously (*20*). We detected amplification products using agarose gel electrophoresis and ethidium bromide staining. We quantified intensity of the ethidium bromide stains using a FluorImager (Molecular Dymanics, Sunnyvale, CA, USA).

Aromatase assay. We determined the catalytic activity of aromatase using the method of Lephart and Simpson (*25*) with minor modifications. Cells were exposed to 54 nM 1β-3H-androstenedione (New England Nuclear Research Products, Boston, MA, USA) dissolved in serum-free (Ultroser SF-free) culture medium and incubated for 1.5 hr at 37°C in an atmosphere of 5% CO_2 and 95% air. All further steps proceeded as reported previously (*20,26*). Aromatase activity was expressed in picomoles of androstenedione converted per hour per milligram cellular protein. We verified the

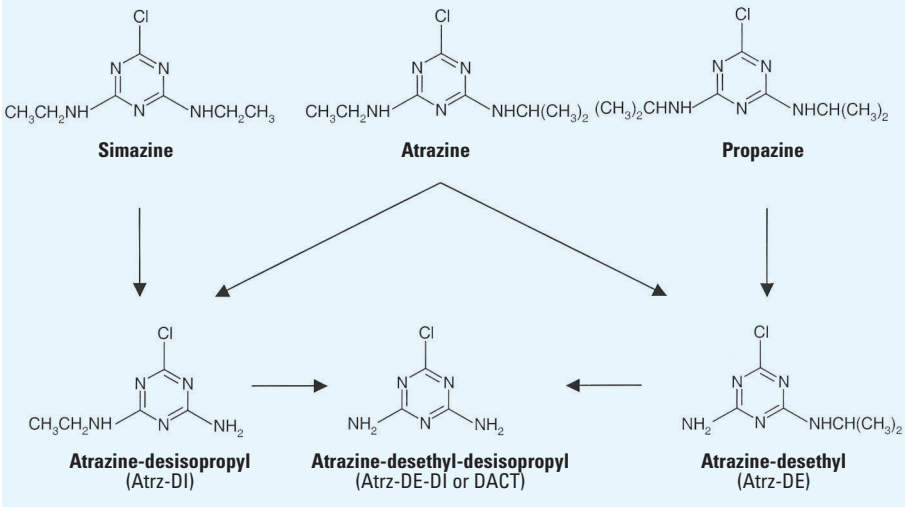

Figure 1. The various routes of metabolism of the 2-chloro-*s*-triazines herbicides atrazine, simazine, and propazine to several common dealkylated metabolites. Each of the three dealkylated metabolites can be further dechlorinated via 2-hydroxylation.

specificity of the aromatase assay based on the release of tritiated water by measuring the production of estrone (the aromatization product of androstenedione), using a ^{125}I-labeled double-antibody radioimmunoassay kit (ICN), and by using 4-hydroxyandrostenedione, an irreversible inhibitor of the catalytic activity of aromatase, to block the formation of tritiated water (*27*).

Carp hepatocyte/vitellogenin production assay. Male carp (*Cyprinus carpio*) hepatocytes were freshly perfused by a two-step retrograde technique, isolated and cultured as described previously in 96-well plates (*21*). Culture conditions included the use of phenol red-free DMEM/F12 medium (Sigma) supplements with 14.3 mM $NaHCO_3$, 20 mM HEPES, 50 µg/L gentamycin, 1 uM insulin, 10 µM hydrocortisone, 2% Ultroser SF and 2 mg/L of the protease inhibitor aprotinin (Fluka, Buchs, Switzerland). Cells were seeded in 96-well plates at a density of 1 × 10^6 cells/mL (180 µL/well). For the estrogenicity studies, we exposed cells to various concentrations of 17β-estradiol (0.06–6 µM) or the triazines and their metabolites (0.3–30 µM), from DMSO stocks. For the antiestrogenicity studies, we used the same triazine concentrations but added them in culture medium containing 100 nM 17β-estradiol (approximate EC_{50}). The final concentration of DMSO did not exceed 0.2% (v/v). As positive controls we included on every plate either a 100 nM 17β-estradiol (for estrogenicity studies) or 0.1, 1.0, and 10 µM tamoxifen, a known estrogen receptor antagonist (for antiestrogenicity studies). All treatments were in sextuplet; each concentration–response experiment was reproduced three times. Exposures were for 6 days. We quantified vitellogenin production by an indirect competitive ELISA, and we determined cell viability as described in detail previously (*21*).

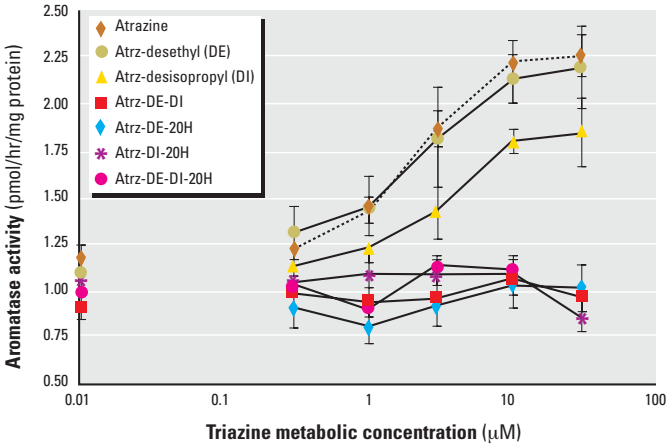

Figure 2. Concentration–response curves for induction of aromatase activity in H295R human adrenocortical carcinoma cells after 24-hr exposure to atrazine and six of its metabolites. Each concentration was tested in quadruplicate.

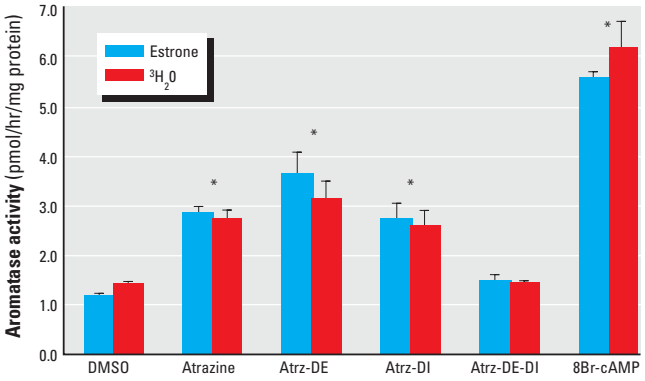

Figure 3. Comparison of aromatase activity based on estrone production of tritiated water release in H295R human adrenocortical carcinoma cells treated for 24 hr with 30 µM atrazine, its metabolites atrazine-desethyl (Atrz-DE), atrazine-desisopropyl (Atrz-DI), and atrazine-desethyl-desisopropyl (Atrz-DE-DI or DACT), or 100 µM 8-bromo-cAMP. Each concentration was tested in quadruplicate.
*Significantly different from control (DMSO)(two-tailed Student *t*-test, $p < 0.05$).

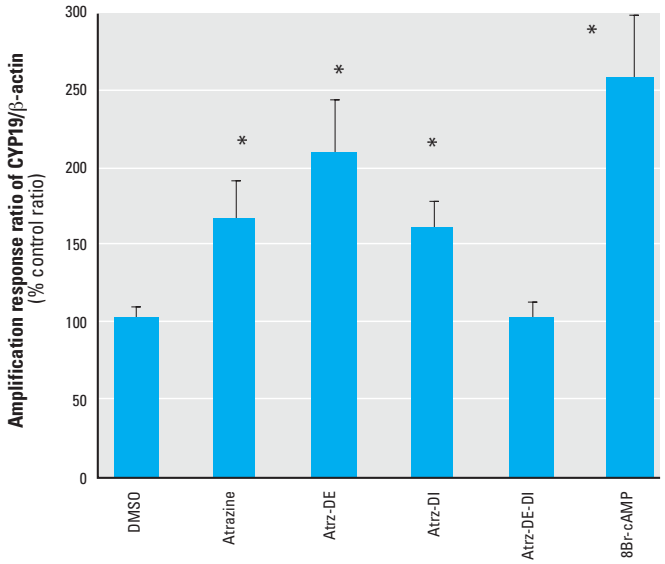

Figure 4. Levels of CYP19 mRNA in H295R human adrenocortical carcinoma cells exposed for 24 hr to DMSO vehicle, 30 µM atrazine, atrazine-desethyl (Atrz-DE), atrazine-desisopropyl (Atrz-DI), or atrazine-desethyl-desisopropyl (Atrz-DE-DI or DACT), or 100 µM 8-bromo-cAMP. Each treatment was tested in triplicate.
*Significantly different from control (DMSO) (two-tailed Student *t*-test, $p < 0.05$).

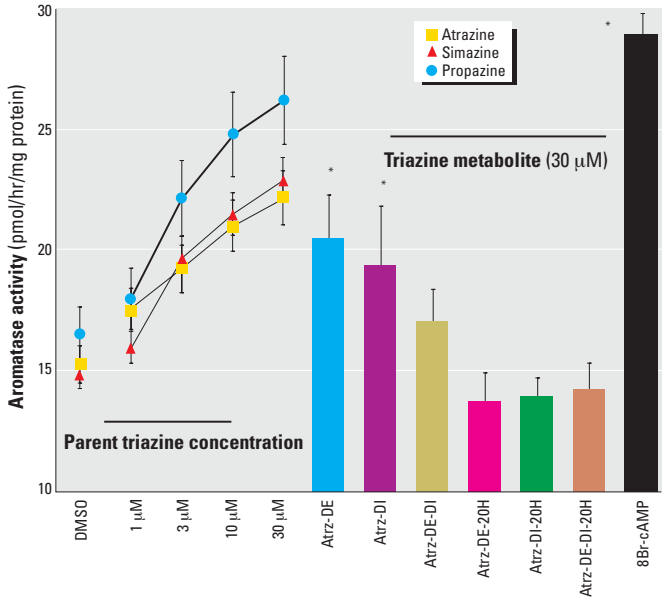

Figure 5. Concentration–response curves for induction of aromatase activity in JEG-3 human placental choriocarcinoma cells after 24 hr exposure to atrazine, simazine, or propazine, and the effect of 24-hr exposure of JEG-3 cells to a single concentration of 30 µM of six triazine metabolites, or 100 µM 8-bromo-cAMP. Each concentration was tested in quadruplicate.
*Significantly different from control (DMSO) (two-tailed Student *t*-test, $p < 0.05$).

Results

Aromatase induction in H295R cells. Atrazine, atrazine-desethyl (Atrz-DE), and atrazine-desisopropyl (Atrz-DI) were able to induce the catalytic activity of aromatase concentration-dependently to an apparent maximum of just over 2-fold (Figure 2). Greater concentrations of these compounds demonstrated slight cytotoxicity (about 20% decrease in MTT reduction at 100 µM). The fully dealkylated metabolite of atrazine—atrazine-desethyl-desisopropyl (Atrz-DE-DI or DACT)—and the metabolites that were hydroxylated at the 2 position of the triazine ring [and thus dechlorinated (Figure 1)] had no effect on aromatase activity (Figure 2). We verified further the differential effects of the triazine compounds on aromatase activity by measuring the ability of the cells to convert androstenedione to estrone. Tritiated water release and estrone production were increased in a 1:1 ratio in cells exposed to atrazine or Atrz-DE, Atrz-DI, and 8Br-cAMP, whereas the metabolite Atrz-DE-DI had no effect on either measurement (Figure 3). The mechanism of induction of aromatase activity appeared to involve the induction of CYP19 mRNA, because atrazine, Atrz-DE, Atrz-DI, and 8Br-cAMP were able to increase mRNA levels for CYP19 relative to control, whereas Atrz-DE-DI had no effect (Figure 4). None of the triazine metabolites could inhibit or enhance the activity of aromatase when added directly to the medium used for the aromatase assay (data not shown). The same was true for the parent triazines (*20*).

Aromatase induction in JEG-3 and MCF-7 cells. Several 2-chloro-*s*-triazine herbicides were able to induce aromatase activity in JEG-3 cells (Figure 5). Atrazine, simazine, and propazine induced aromatase activity concentration-dependently, producing statistically significant increases in activity above control at concentrations above 1 µM (Student *t*-test, $p < 0.05$). Among the metabolites of atrazine tested at 30 µM, only Atrz-DE and Atrz-DI significantly increased aromatase activity above control. The most noticeable difference between JEG-3 and H295R cells is the basal activity of aromatase, which was at least an order of magnitude greater in JEG-3 cells (about 15 pmol androstenedione/hr/mg cellular protein) than in H295R cells (about 1–1.5 pmol androstenedione/hr/mg cellular protein); also, basal activity in JEG-3 cells was inducible by only 2-fold after 24-hr exposure to 100 µM 8Br-cAMP, whereas aromatase activity was inducible by over 5-fold in H295R cells. In MCF-7 cells, basal aromatase activity was undetectable, and neither 8Br-cAMP, atrazine, simazine, nor propazine was able to induce the activity to detectable levels, under our culture and assay conditions; the same was true for mRNA levels (data not shown).

Vitellogenin production in carp hepatocytes. Vitellogenin concentrations in unexposed or DMSO-exposed male carp hepatocytes were undetectable. A lowest-observed-effect concentration of 17β-estradiol of about 2 nM produced a detectable amount of vitellogenin of about 100–400 ng/mg cellular protein; the EC_{50} of 17β-estradiol induced vitellogenin concentrations to 4,000–6,000 ng/mg protein. Coexposure of hepatocytes to 100 nM 17β-estradiol and 0.1, 1, or 10 µM tamoxifen inhibited 17β-estradiol-induced vitellogenin synthesis by 54%, 89%, and 91%, respectively. The readily aromatizable androgens testosterone and 17α-methyltestosterone did not induce vitellogenin synthesis at concentration between 0.6 nM and 1 µM (6-day exposures), indicating that aromatase activity is either very low or not present in male carp hepatocytes in primary culture under our conditions. Exposure of male carp hepatocytes to various concentrations (0–30 µM) of the triazines or their metabolites did not significantly induce vitellogenin production (Figure 6A). The only exception was a slight, but statistically significant ($p < 0.05$) and concentration-dependent estrogenic response by Atrz-DE-DI (DACT), which increased vitellogenin production from 2% of the response by 100 nM 17β-estradiol at 1 µM (not shown) to about 8% of the response by 100 nM 17β-estradiol at 30 µM (Figure 6A). None of the compounds could produce a concentration-dependent antiestrogenic response in the presence of 100 nM 17β-estradiol (Figure 6B).

Discussion

We recently reported that several chloro-*s*-triazine herbicides induce the catalytic activity and mRNA expression of human aromatase *in vitro* in H295R adrenocortical carcinoma cells (*20*). The present study extends these observations by demonstrating that atrazine, simazine, propazine, and two metabolites shared by these Atrz-DE and Atrz-DI—were able to induce aromatase activity in H295R cells, whereas the fully dealkylated metabolite Atrz-DE-DI (DACT) and the three hydroxylated metabolites of atrazine were not active. In addition, the compounds that induced aromatase activity in H295R cells also induced this activity in JEG-3 cells, although with lesser efficacy. A difference between the two cell lines is that JEG-3 cells exhibited a 15-fold greater basal aromatase activity than H295R cells, and inducibility by 8Br-cAMP was lower (< 2-fold) than in H295R cells (over 5-fold). Thus, the relatively high level of basal aromatase gene expression in JEG-3 cells and relatively low inducibility by the cAMP analog partly explains the lesser response to the triazines.

MCF-7 cells did not exhibit aromatase activity in this study, nor did they respond to induction by cAMP analogs or triazine herbicides. The expression of aromatase in MCF-7 cells has been the subject of conflicting reports. Although many studies have not detected aromatase activity in MCF-7 cells (*28*), some report the presence of low aromatase activity (*29–31*) and of stimulation of estrogen-receptor–mediated cell proliferation by androgens in this cell line (*30*). The expression of aromatase in MCF-7 cells is poorly understood, and although at least one study reported stimulation of this enzyme by cAMP (*31*), we have not been able to stimulate MCF-7 cells to express detectable levels of activity using 8Br-cAMP or forskolin. The above suggests that major qualitative differences exist in characteristics among batches of MCF-7 cells in culture, which may complicate the use of this cell line as an *in vitro* screening tool for effects

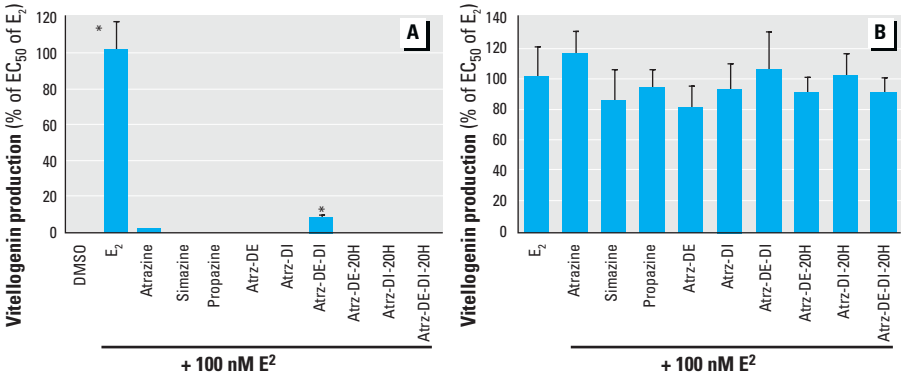

Figure 6. Exposure of male carp hepatocytes to triazines and metabolites. (*A*) Effect of 100 nM estradiol (E_2) or 30 µM of the three parent triazines and six metabolites on vitellogenin production in freshly isolated male carp hepatocytes. (*B*) Effect of a coexposure of freshly isolated male carp hepatocytes to 100 nM E_2 and 30 µM of each of the three parent triazines or their six metabolites individually, on the induction of vitellogenin synthesis. Each concentration was tested in quadruplicate.
*Significantly different from control (DMSO) (two-tailed Student *t*-test, $p < 0.05$).

of androgens and estrogens, or effects of xenobiotics on steroidogenic and/or steroid metabolizing enzymes. In any case, our findings indicate that, unlike in cell systems in which the expression of aromatase activity is clearly cAMP-dependent (H295R and JEG-3), triazine herbicides do not induce aromatase activity in MCF-7 cells, in which this expression is relatively refractory to cAMP.

Regarding a structure–activity relationship for aromatase induction by the different triazine compounds, it appeared that the relatively lipophilic parent triazines and monodealkylated metabolites were active, whereas the more hydrophilic fully dealkylated and 2-hydroxylated (dechlorinated) metabolites were inactive. These results indicate that biokinetic factors such as metabolism may play a considerable role in the biologic activity of triazine herbicides. Whether the structure-activity relationship observed for aromatase induction *in vitro* corresponds with the potential to increase estradiol levels or cause estrogen-mediated toxicities *in vivo* is not certain. Indeed, atrazine, simazine, and propazine appear to have similar effects on mammary tumor incidences *in vivo* (*32*), but toxicologic information on the fully dealkylated metabolite of the triazines is insufficient to make a judgment. To substantiate the aromatase induction hypothesis, additional experimental evidence is required to determine whether aromatase induction occurs *in vivo* and in which target tissues this induction would take place. Given the recent evidence that plasma estradiol and estrone levels are increased in atrazine-treated male Wistar rats (*33*), it is apparent that the presence of ovarian aromatase is not essential for the effects of atrazine. The further observation that estrone levels appear to be preferentially increased *in vivo* (*33*) may indicate a tissue-specific effect on aromatase. If aromatase induction is shown to play a role *in vivo*, it could be hypothesized that the induction would occur in tissues that contain relatively greater levels of androstenedione than testosterone as precursor—tissues such as adrenal cortex and adipose.

The lack of response of male carp hepatocytes to the triazines and their metabolites is consistent with the observed lack of interaction of these compounds with the estrogen receptor and inability to cause estrogen receptor-mediated responses *in vitro* (*13–15*). In addition, measurable aromatase activity was not found in the hepatocytes because readily aromatizable androgens were not able to elicit vitellogenin induction. However, we do not rule out the presence of low levels of aromatase activity in male carp hepatocytes in primary culture, and future investigations will be needed to address this question.

In conclusion, the effects of the triazine herbicides and some of their metabolites on aromatase activity may provide a partial explanation for the observed increases in plasma estradiol concentrations in female Sprague-Dawley rats (*7*) and estradiol and estrone concentrations in male Wistar rats (*33*) exposed to atrazine, together with the observed estrogen-mediated toxicities *in vivo* (*7*). Future studies are needed to investigate the inducibility of aromatase by the various triazine herbicides and their metabolites *in vivo*, and compare the developed structure-activity relationship for induction to *in vivo* estrogenic toxicities and to the *in vitro* results of the present study.

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salt solution (HBSS), at 900*g* for 20 min. Pellets were resuspended at stepped suspension concentrations from 10/13 mg/mL to 10 mg/mL, except SC1, which was adjusted to one-tenth the concentration of other samples. These suspensions were stored at 4°C.

Cell isolation. We obtained heparinized blood from healthy donors by venipuncture and diluted it 1:1 in HBSS. We isolated monocyte–lymphocyte fractions by Ficoll density centrifugation and plated them in 9-cm-diameter plastic tissue culture dishes for monocyte adherence (*23*). We cultured the adhering cells for 9 days in RPMI1640 HEPES modification (Sigma Chemical Co., St. Louis, MO, USA) with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. This culture medium was changed every 2 days. Adherent cells were separated after 6 days, and suspended in serum-free RPMI1640.

Chemiluminescence measurements. The method of measurement of lucigenin-dependent CL from 6-day-old human monocyte-derived macrophages exposed to various mineral fibers has been described (*24*): The lucigenin responses increased with the increasing age of cultures over 6 days, and Nyberg and Klockars (*24*) obtained a correlation between lucigenin-dependent CL and superoxide production measured with the cytochrome C reduction assay at 6 days of culture.

The isolated cells (1×10^5 cells) were transferred into a luminometer tube containing mineral sample suspension (65 µL), 10% FBS, 0.1 mM lucigenin, and in some experiments 1,000 U/mL superoxide dismutase (SOD). The final volume of each tube was 1 mL. The light emission of each sample was detected at 15-min intervals with a luminescence reader (ALOKA BLR-201; Mitaka, Tokyo, Japan). We measured all samples including the negative control (no fiber) with the same cell suspension at 10-sec intervals. We performed all reactions at 37°C in RPMI 1640, each measurement 4 times.

Statistical analysis. We analyzed the ability to induce CL per fiber of each sample as described previously (*20*). Briefly, we examined the relation between the estimated number of fibers administered and CL response by linear regression. The slope (β_1) of the regression line was taken as a measure of the ability to induce CL per fiber. We excluded the data of β_1 for $r^2 < 0.9$. We also examined the relation between fiber size and ability to induce CL by linear regression, and calculated the increase in the rate of induction with two β_1. We examined the time course of the increase in the ability to induce CL by power regression. Finally, we examined the relation between fiber size and increased ability to induce CL using linear regression.

Results

The time course of the ability to induce CL per fiber (β_1). We tested the CL response of all JFM preparations and controls at constant rotation every 15 min by using a stock of cells in suspension. We needed β_1 to compare the CL response of each sample at a value not related to the number of fibers administered. Table 1 shows β_1 and r^2. All fiber samples except for WO1 induced a CL response in a dose-dependent manner. Each response was almost completely inhibited by SOD, which is a superoxide scavenger (data not shown). WO1 was excluded in subsequent analyses because its CL response increased rectilinearly and the linearity of its dose response was low (Table 1). Moreover, we also excluded the β_1 data for $r^2 < 0.9$ at each measurement time.

As shown in Figure 1, each JFM standard reference sample produced a sigmoid-type increase in β_1. The pattern of increase in β_1 for each sample was similar, although the values differed.

The similarity of the increase in β_1 to JFM samples. We calculated the rate of increase in β_1 to demonstrate the similarity of the response pattern to various mineral fibers. Table 2 shows the rate for each time point. Although each rate of increase was different at 15–30, the kinetics of the rate were relatively similar in these cases. As shown in Figure 2, the rate of β_1 decreased for the power regression line. Table 3 shows constants and the r^2 of the power regression lines. These comparisons showed the similarity of each CL response more clearly. However, the thickest fiber (RF3) and the thinnest fiber (TO1) had slightly lower correlations than other samples. The rate of RF3 was low in the acute phase, and the rate of TO1 was high in the acute phase.

The relationship between β_1 and fiber length. Figure 3 shows a representative time-dependent relation between geometric-mean length and β_1, used to examine the effect of fiber length on CL response. The results are shown in Table 4 with constants and the r^2 of the regression lines. A close correlation existed between length and β_1 at each time point, although four samples under approximately 6 µm in length (SC1, PT1, MG1, and TO1) had a low β_1. Therefore, a further close correlation existed between length and β_1 with samples > 6 µm in length (GW1, RW1, RF1, RF2, RF3, SC1, and PT1) after

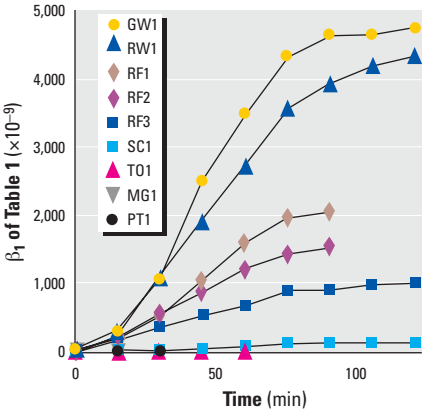

Figure 1. Time course of ability to induce CL from macrophages exposed to various mineral fibers (β_1 of Table 1). Each point is the mean from four measurements. The defects are the cases where r^2 is < 0.9 (shown in Table 1).

Table 1. Constants and r^2 of the regression lines for CL and estimated number of fibers.

Time[a]	0		15		30		45		60		75		90		105		120	
(min)	β_1[b]	r^2[c]	β_1	r^2	β_1	r^2	β_1	r^2	β_1	r^2	β_1	r^2	β_1	r^2	β_1	r^2	β_1	r^2
GW1	15.34	0.110	280.0	0.960	1,052	0.958	2,492	0.942	3,489	0.927	4,333	0.941	4,643	0.920	4,642	0.947	4,735	0.940
RW1	9.869	0.406	345.4	0.966	1,072	0.979	1,920	0.961	2,735	0.959	3,581	0.952	3,946	0.924	4,209	0.937	4,352	0.938
MG1	0.436	0.339	21.97	0.972	66.20	0.902	112.1	0.818	153.8	0.815	182.0	0.819	179.8	0.798	162.7	0.761	142.2	0.735
RF1	0.664	0.020	168.3	0.920	517.9	0.992	1,021	0.985	1,601	0.975	1,955	0.952	2,035	0.928	1,877	0.865	1,580	0.780
RF2	–0.75	0.028	214.3	0.985	562.2	0.988	893.9	0.967	1,219	0.944	1,447	0.921	1,538	0.916	1,439	0.897	1,327	0.848
RF3	7.742	0.422	177.0	0.978	371.0	0.992	539.1	0.979	669.1	0.937	910.5	0.982	914.7	0.970	997.0	0.970	1,021	0.967
PT1	–0.03	0.162	2.130	0.971	6.100	0.940	9.600	0.896	11.40	0.818	10.40	0.685	8.900	0.608	6.700	0.484	4.800	0.373
SC1	0.307	0.228	7.600	0.917	24.40	0.992	49.20	1.000	77.80	0.996	110.6	0.996	123.1	0.994	128.9	0.992	132.4	0.990
TO1	–0.03	0.203	1.520	0.977	3.200	0.974	7.000	0.988	11.00	0.913	12.00	0.822	11.10	0.738	9.400	0.662	7.300	0.607
WO1	–0.29	0.072	–5.69	0.450	–2.90	0.041	8.900	0.124	28.20	0.338	61.80	0.580	95.30	0.688	118.8	0.765	137.1	0.786

[a]Time after administration; CL responses of 54 samples were measured in constant rotation at 15–min intervals with the same stock suspension of cells. [b]β_1 ($\times 10^{-9}$) is the slope of the regression line for the estimated number of fibers administered and CL response with 5 concentrations and a duplicate negative control. The CL response is the mean value of the four measurements. [c]Square of the correlation coefficient of the regression line.

30 min. The relation between length and β_1 lasted from the acute phase of the reaction to 120 min.

The relationship between β_1 and fiber width. The World Health Organization (WHO) classifies mineral fibers based on length, width, and the aspect ratio of the fiber (*25*). Figure 4 shows the relation between geometric-mean width and β_1 at 15 and 45 min. The results are shown in Table 5 with constants and the r^2 of the regression lines. As shown in Figure 4 and Table 5, we observed a close correlation between width and β_1 for eight samples < 1.8 μm in width at 15 min (r^2 = 0.8766); however, this relationship did not continue (r^2 at 45 min = 0.5138). β_1 correlated with width more than with length at 15 min, but it correlated with length more than with width after 30 min.

The relationship between increase rate of β_1 and width. We examined the relationship between rate of β_1 and fiber width to demonstrate the effect of the width from 15 to 60 min. Figure 5 shows a representative relationship between rate of β_1 and fiber width. The results are shown in Table 6 with constants and the r^2 of the regression lines. Although the tendency of this relationship at 15–30 min resembles that of β_1 and width at 15 min (Figure 4A), we observed a correlation at 30–45 min [r^2 = 0.5309 (Figure 5B)] and at 45–60 min [r^2 = 0.7473 (Figure 5C)]. However, a slope of the regression line decreased over the time course. Moreover, as shown in Table 2, the increase of β_1 was similar in each sample after 60 min. Therefore, we saw no correlation at 60–75 min (Table 6).

The relationship between increase rate of β_1 and length. We also examined the relation between rate of β_1 and fiber length. The correlation between these could not be recognized at any time point (data not shown).

The relationship between CL response and fiber sample weight. The relationship between sample weight and CL response at 45 min is shown in Figure 6A. These data were the most rectilinear for the dose–response curve in the time-course measurement. Table 7 shows a slope of regression line of the dose–response curves in mass concentration. MG1 had the highest level, and GW1 and RF3 had the lowest level. However, the linearity of dose–response curves did not continue in some samples. The relationship between sample weight and CL response at 120 min is shown in Figure 6B as reference. The dose–response curve of some samples was saturated at various levels. Short fibers tend to saturate the dose–response curve at low dosage.

Table 2. Time course of the rate of increase in β_1, in 15-min intervals.

No.[a]	1 (15–30)	2 (30–45)	3 (45–60)	4 (60–75)	5 (75–90)	6 (90–105)	7 (105–120)
GW1	3.757	2.369	1.400	1.242	1.072	1.000	1.020
RW1	3.104	1.791	1.424	1.309	1.102	1.067	1.034
MG1	3.012	—[b]	—	—	—	—	—
RF1	3.078	1.971	1.569	1.221	1.041	—	—
RF2	2.623	1.590	1.364	1.186	1.063	—	—
RF3	2.096	1.453	1.241	1.361	1.005	1.090	1.024
PT1	2.845	—	—	—	—	—	—
SC1	3.216	2.013	1.582	1.421	1.113	1.047	1.027
TO1	2.131	2.165	1.572	—	—	—	—
Average	2.874	1.907	1.450	1.290	1.066	1.051	1.026
SD	0.498	0.296	0.120	0.082	0.036	0.033	0.005

[a]Time-course order of the rate of increase in β_1. The rates were calculated between continuing two data points, in minutes. For example, the values at 1 are β_1 at 30 min divided by the β_1 at 15 min. [b]The defects were the cases where r^2 < 0.9.

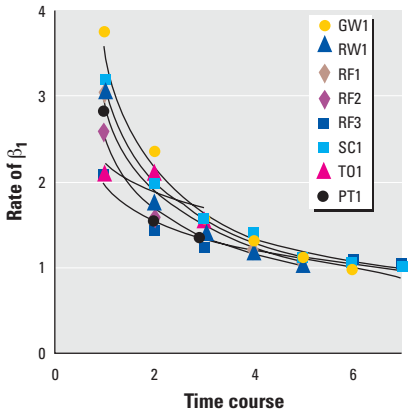

Figure 2. Time course of the increase in β_1. The defects are the cases where r^2 is < 0.9. The curves were approximated by power regression.

Table 3. Constants and the r^2 of the power regression lines for time course of the rate of increase in β_1.

Fibers	A[a]	B[a]	r^{2b}	n^c
GW1	3.601	–0.720	0.961	7
RW1	2.849	–0.563	0.970	7
MG1	–	–	–	1
RF1	3.123	–0.669	0.9959	5
RF2	2.512	–0.550	0.9829	5
RF3	1.983	–0.356	0.8972	7
PT1	–	–	–	1
SC1	3.136	–0.693	0.9889	7
TO1	2.240	–0.245	0.5681	3

[a]Constants of the power regression line for the time course of the rate in Figure 2. For convenience, numbering was used to estimate the regression line. Equation, $Y = AX^B$; Y = rate of increase in Table 2, X = numbering of the rate in Table 2. [b]Square of the correlation coefficient of the power regression line. Though constant, A changes with the numbering; constant B and r^2 are fixed. [c]Effective number.

Discussion

The results of the present study demonstrate the time course and rate of the induction of lucigenin-dependent CL in human monocyte-derived macrophages for various man-made and natural mineral fibers. Moreover, we examined the time-dependent relationships between fiber size and these parameters. Even when the dosed number of fibers differed for each sample, the ability to induce CL per fiber could be approximated using our analysis.

Many intrapleural studies led to the conclusion that the fibrous shape of asbestos dust particles is the cause of their carcinogenicity in humans and that basically all types of elongated dust particles such as mineral and vitreous fibers can induce tumors if they are sufficiently long, thin, and durable in the tissue (*10,26*). If this conclusion is true, common reactivity in the mechanism of tumor induction should exist between asbestos and mineral and vitreous fibers. Numerous studies have suggested that ROS may underlie the pathogenesis of asbestos-related lung diseases (*11,27*). However, amphibole asbestos, which includes iron in its fibers, plays a special role in ROS-mediated pathology because it catalyzes the generation of the reactive hydroxyl radical from hydrogen peroxide (*11,28,29*). In asbestos, the hydroxyl radical can alter various biologic effects (*11–13*). In biologic systems, superoxide usually acts as the reductant producing Fe^{2+}, which rapidly decomposes hydrogen peroxide to hydroxyl radicals (*29,30*). The action of superoxide makes a chain of reactions in which the net process converts hydrogen peroxide to the hydroxyl radical (*29,31*). Paradoxically, superoxide activity may decide hydroxyl radical activity *in vivo*, because hydrogen peroxide has always been made *in vivo* if Fe^{3+} exists in close proximity. Various mineral fibers cause a significant increase in the release of superoxide from macrophages (*18,19*). Moreover, tumorigenic fibers do not always have hydroxyl radical activity *in vitro* (*14*). Silicon carbide fibers, one type of tumorigenic fiber, have no hydroxyl radical activity (*14*). Our findings here suggest that macrophages have common superoxide reactivity for various types of fiber and that the activity of superoxide from macrophages has an important role in biologic effects, depending on fiber length.

In early animal intraperitoneal studies, it was suggested that the induction of pleural sarcoma increased with the length of fibers with diameters < 1.5 μm (*32*). However, a

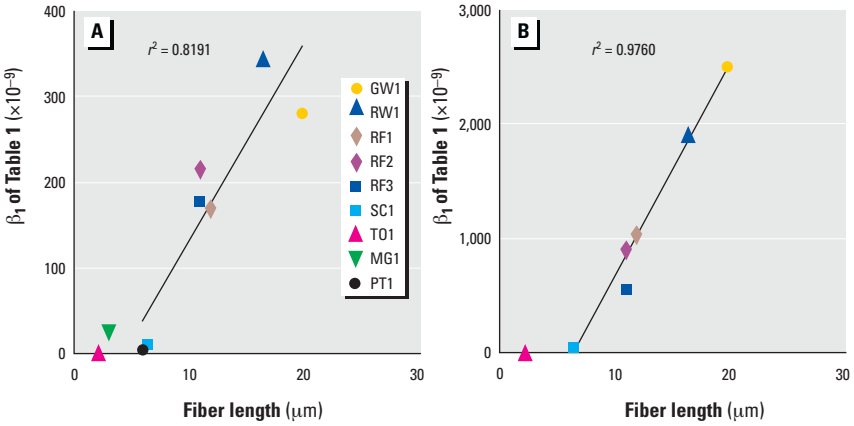

Figure 3. The relationship between geometric-mean length and β_1 of Table 1. The line is a regression line for samples > 6 μm in length. The data for r^2 < 0.9 were excluded. (*A*) Data at 15 min; this correlation is the lowest. (*B*) Data at 45 min; this correlation is the highest.

Table 4. Constants and the r^2 of the regression lines for β_1 of Table 1 and fiber length.

Time[a]	0	15	30	45	60	75	90	105	120
A[b]	0.080	2.027	68.27	149.7	211.2	332.1	360.7	367.3	376.2
B[b]	–4.062	–6.286	–259.1	–700.4	–983.5	–2,200	–2,423	–2,456	–2,509
r^{2c}	0.677	0.852	0.906	0.916	0.907	0.965	0.957	0.946	0.943
n^d	9	9	9	7	7	6	6	4	4
A[e]	1.068	23.04	82.77	186.8	263.2	332.1	360.7	367.3	376.2
B[e]	–7.910	–102.2	–465.1	–1,241	–1,741	–2,200	–2,423	–2,456	–2,509
r^{2f}	0.738	0.819	0.937	0.976	0.962	0.965	0.957	0.946	0.943
n	7	7	7	6	6	6	6	4	4

[a]Time after administration (min). [b]Analysis for nine samples. A and B are constants of the regression line for β_1 and fiber length. Equation: Y = AX + B; Y = β_1 of Table 1, X = geometric-mean length of fibers, A, B = constants (× 10^{-9}). [c]Square of the correlation coefficient of the regression line with whole samples. [d]Effective number. The data < 0.9 in r^2 of Table 1 were excluded, except for time 0. The data of time 0 are reference data. All effective data after 75 min were samples > 6 μm in length. [e]Analysis for seven samples > 6 μm in length. A,B and equation were the same as *b*. [f]Square of the correlation coefficient of the regression line with samples > 6 μm in length.

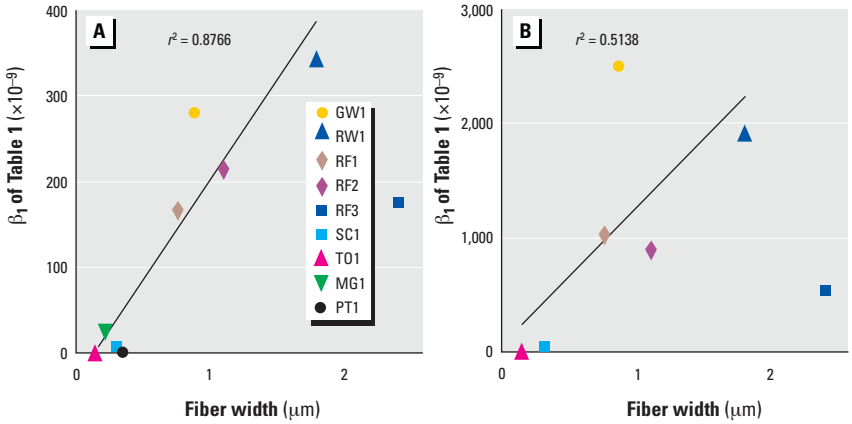

Figure 4. The relationship between geometric-mean width and β_1 of Table 1. The continuous line is a regression line with samples < 1.8 μm in width. (*A*) Data at 15 min; this correlation is the highest. (*B*) Data at 45 min.

Table 5. Constants and the r^2 of the regression lines for β_1 of Table 1 and fiber width, except RF3.

Time[a]	0	15	30	45	60	75	90	105	120
A[b]	5.930	232.8	719.7	1,196	1,686	1,917	2,134	2,429	2,519
B[b]	-0.911	-32.26	-88.98	68.85	119.7	426.2	386.6	580.1	571.3
r^{2c}	0.311	0.877	0.775	0.514	0.515	0.387	0.407	0.545	0.556
n^d	8	8	8	6	6	5	5	3	3

[a]Time after administration (min). [b]Analysis for eight samples < 1.8 μm in width. A and B are constants of the regression line for β_1 and fiber width. Equation: Y = AX+B; Y = β_1 of Table 1, X = geometric-mean width of fibers, A,B = constants (× 10^{-9}). [c]Square of the correlation coefficient of the regression line. [d]Effective number. The data < 0.9 in r^2 of Table 1 were excluded, except for time 0. The data of time 0 are reference data.

relation between ROS and fiber width has not been shown. We also tried to analyze the effect of fiber width on the ability to induce CL. Our results showed that wide fiber (a width of 2.4 μm) has a low ability to induce CL and that thin fibers cause a large acceleration in the induction of CL in the acute phase. However, our findings suggest that the superoxide-mediated biologic effect of width is weak because the effect of width on the ability to induce CL was smaller than that of length. If a biologic effect of width does exist, thin fibers may be stronger than thick fibers of the same length.

WHO has classified fibers > 5 μm long, < 3 μm diameter, with an aspect ratio > 3:1 (*25*). Our findings suggest that many airborne WHO fibers induce superoxide release from macrophages depending on fiber length.

Long asbestos fibers are more effective than short fibers in eliciting the release of superoxide from macrophages (*16*). However, the molecular mechanism by which asbestos may augment the release of oxygen metabolites from phagocytic cells is unclear. One hypothesis is that oxidant release occurs nonspecifically during "frustrated" phagocytosis by alveolar macrophages and polymorphonuclear leukocytes that are unable to ingest long asbestos fibers completely (*33*). However, our findings do not support this hypothesis, because the time-dependent pattern (sigmoid type) and increase of ability to induce CL were similar for each sample except wollastonite (Figures 1,2). These findings suggest that though the release of superoxide from macrophages occurs nonspecifically for many mineral fibers, the intensity had already been decided when fibers were phagocytosed to some extent. If the release of superoxide occurs during "frustrated" phagocytosis, the intensity of that of short fibers should decrease with the advance of phagocytosis.

We speculated as to the reason why the ability to induce CL increased with fiber length when samples were longer than approximately 6 μm. The regular transition in the rate to induce CL in each sample suggests that the intensity of the CL response is decided at the initial stage of phagocytosis. However, it cannot be considered that macrophages recognized fiber length at the initial stage of phagocytosis. In observations

by optical microscope, short fibers were perpendicularly phagocytosed, and long fibers were often tangentially phagocytosed (data not shown). Therefore, we speculated that tangential phagocytosis has a stronger effect on the ability to induce CL than perpendicular phagocytosis. If this speculation is true, a cause of the enhanced ability to induce CL may be the increase in the tangential phagocytic rate with lengthening of fiber. Moreover, we speculated that tangential phagocytosis shifts to perpendicular phagocytosis with fibers under approximately 6 μm in length.

In general, many experimental protocols have been conducted based on the mass concentration of fiber samples. Therefore, we also show the CL response per sample weight (Figure 6A) to allow comparison with other experimental results. In comparison by mass concentration, our data showed that the CL response is weak in both the short samples and samples such as glass wool and rock wool, which have low fiber numbers per unit weight. Mass concentration study of glass wool and rock wool showed no significant increase in tumor incidence in rats (*4,5*). The data in Figure 6A are consistent with these *in vivo* results. Moreover, a durable special application fiber glass (MMVF33, 106 fibers/cc > 20 μm) induced lung fibrosis and a single mesothelioma in hamsters; however, insulation fiber glass (MMVF10a, 151 fibers/cc > 20 μm) did not induce lung fibrosis or tumors (*34*). The data in Figure 1 are consistent with the finding that the glass fiber is not inert.

Previous studies with various mineral particles have suggested that the fibrous geometry of particulates is of critical importance in the generation of superoxide from macrophages (*16,18,35*). For example, for amosite asbestos, dramatic enhancement of release of superoxide has been found with long fibers but not short ones (*35*). The distribution of length of the long fibers (50% > approximately 14 μm long) is similar to that of RF1 (mean length 12.0 μm), and the distribution of length of the short fibers (10% > approximately 10 μm long) is similar to that of MG1 (mean length 3.0 μm). Therefore, our data on the relationship between fiber length and ability to induce CL are consistent with the asbestos data. Moreover, our findings suggest that this relationship continues over the time course without effect of fiber clearance. In contrast, murine peritoneal macrophages exposed to equal numbers of short and long crocidolite asbestos fibers exhibited comparable hydrogen peroxide release (*36*). However, the mean length of the long crocidolite fiber was 5.4 μm, and the mean length of short fiber was 1.2 μm. Our finding that the ability to induce CL was similar among fibers under approximately 6 μm in length was also consistent with the hydrogen peroxide data.

These assays were performed with suspended cells over a time course of 2 hr. Many previous published studies of effects of asbestos and mineral fibers on oxidant production from alveolar macrophages have used cells in suspension. However, many studies of the effect of fiber length on oxidant production and using monocyte-derived macrophages have used adherent cells. For some applications, suspended cells work better than adherent cells for comparing the response of cells. First, the number of cells in each vial will be identical with that of cell suspension. Second, the cells will have diffuse contact with the fibers. We believe that this advantage contributes to linearity of the dose–response curve of CL response. Finally, the cells may smoothly phagocytose the fiber. We consider that these advantages help reduce experimental error.

One problem is whether wollastonite is an exception. Although WO1 was excluded in our analyses, r^2 and β_1 of WO1 increased

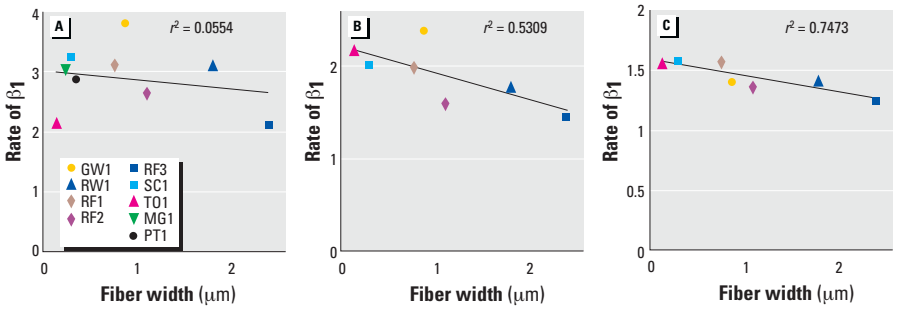

Figure 5. The relationship between geometric-mean width and rate of β_1. The continuous line is a regression line on the whole. (*A*) Data at 15–30 min. (*B*) Data at 30–45 min. (*C*) Data at 45–60 min; this correlation is the highest. The data for $r^2 < 0.9$ were excluded.

Table 6. Constants and the r^2 of the regression lines for the rate of increase in Table 2 and fiber width.

No.[a]	1	2	3	4	5	6	7
A[b]	–0.161	–0.290	–0.139	0.012	–0.029	0.029	0.001
B[b]	3.016	2.213	1.597	1.276	1.101	1.012	1.025
r^{2c}	0.055	0.531	0.747	0.010	0.316	0.505	0.044
n[d]	9	7	7	6	6	4	4

[a]Time-course order of the rate of increase in Table 2. [b]Constants of the regression line for the rate in Table 2 and fiber width. Equation: Y = AX+B; Y = the rate in Table 2, X = geometric-mean width of fibers. [c]Square of the correlation coefficient of the regression line. [d]Effective number. The data < 0.9 in r^2 of Table 1 were excluded.

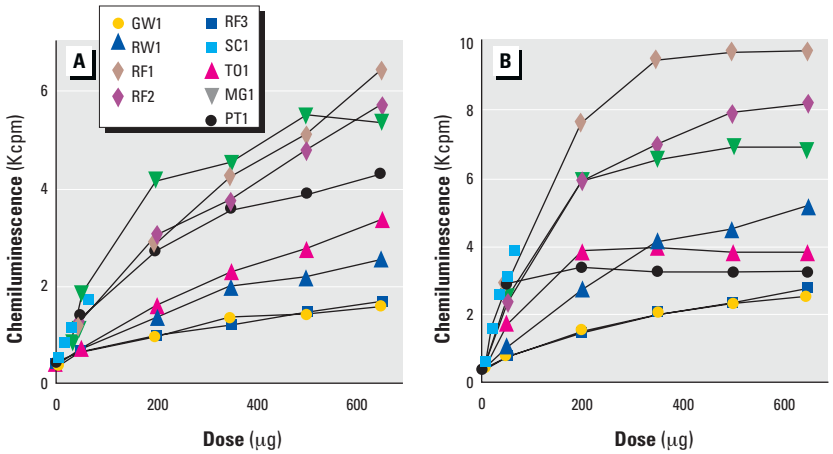

Figure 6. The relationship between sample weight and CL response. (*A*) Data at 45 min. (*B*) Data at 120 min. The data of WO1 were excluded.

Table 7. A slope of regression line of each dose–response curve at 45 min in mass concentration.

Sample	GW1	RW1	MG1	RF1	RF2	RF3	PT1	SC1	TO1
Slope (CL/mg)	1.744	3.264	7.285	8.980	7.777	1.887	5.658	20.18	4.495

Each r^2 was the same as that of Table 1.

over the time course (Table 1). The response for WO1 may be retarded; however, our data are not sufficient to define WO1 as an exception.

In conclusion, it is suggested that macrophages nonspecifically induce superoxide for various fiber types depending on fiber length. Although the generation of hydroxyl radical may be the most important difference between amphibole asbestos and other mineral fibers, superoxide is a tumor promoter and is involved in the generation of hydroxyl radical. Our findings suggested that even inert mineral fibers were not safe if the conditions of durability, clearance, and respirability are satisfied. Our findings have also revealed important differences from the hypothesis that oxidant release occurs during "frustrated" phagocytosis. A remaining problem is to elucidate the reasons why macrophages have high superoxide activity for long fibers.

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Air Pollution and Retained Particles in the Lung

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Epidemiologic evidence associates particulate air pollution with cardiopulmonary morbidity and mortality. The biological mechanisms underlying these associations and the relationship between ambient levels and retained particles in the lung remain uncertain. We examined the parenchymal particle content of 11 autopsy lungs from never-smoking female residents of Mexico City, a region with high ambient particle levels [3-year mean PM_{10} (particulate matter ≤ 10 μm in aerodynamic diameter)= 66 μg/m^3], and 11 control residents of Vancouver, British Columbia, Canada, a region with relatively low levels (3-year mean PM_{10} = 14 μg/m^3). Autopsy lungs were dissolved in bleach and particles were identified and counted by analytical electron microscopy. Total particle concentrations in the Mexico City lungs were significantly higher [geometric mean = 2,055 (geometric SD = 3.9) × 10^6 particles/g dry lung vs. 279 (1.8) × 10^6 particles/g dry lung] than in lungs from Vancouver residents. Lungs from Mexico City contained numerous chain-aggregated masses of ultrafine carbonaceous spheres, some of which contained sulfur, and aggregates of ultrafine aluminum silicate. These aggregates made up an average of 25% of the total particles by count in the lungs from Mexico City, but were only rarely seen in lungs from Vancouver. These observations indicate for the first time that residence in a region with high levels of ambient particles results in pulmonary retention of large quantities of fine and ultrafine particle aggregates, some of which appear to be combustion products. *Key words*: air pollution, environmental exposure, particles, pulmonary retention. *Environ Health Perspect* 109:1039–1043 (2001). [Online _____]

http://ehpnet1.niehs.nih.gov/docs/2001/109p1039-1043brauer/abstract.html

Epidemiologic studies indicate that current levels of particulate air pollution are associated with adverse health outcomes, including increased cardiopulmonary mortality (*1,2*). Although evidence suggests that short-term impacts of particulate air pollution are displacing deaths by more than months, of greater public health significance is the potential for long-term impacts that may shorten lives by years or that may lead to chronic cardiopulmonary morbidity. Several prospective cohort studies provide evidence of such long-term effects, including associations between ambient particles and lung cancer (*3–5*). Whereas acute effects may be limited to those individuals with existing cardiopulmonary disease, chronic exposures may affect a much larger proportion of the exposed population. Although the epidemiologic evidence points to a causal relationship with particles originating in combustion processes, the biological mechanism(s) as well as the exact types and sizes of particles involved are the subjects of intensive investigation. One hypothesis is that the ultrafine particle size fraction is responsible for the epidemiologic observations (*6*). This hypothesis is partly based on the fact that the majority of atmospheric particles, by number, are in the ultrafine mode. These particles, produced in combustion processes, are likely to contain condensates of toxic metals and surface acidity. In animal models, ultrafine particles appear to induce an intense inflammatory reaction and are believed to be translocated to the pulmonary interstitium in large numbers (*7,8*).

Despite the interest in the topic, little is known of the types, sizes, and locations of ambient atmospheric particles in human lungs. Direct measurements of deposited particles in humans are difficult, but animal models show that virtually all types of inhaled particles can be translocated across the alveolar epithelium to the interstitium, from which location they are cleared slowly or not at all (*9*). Analysis of lung parenchymal particle burden can thus provide an indication of the types and numbers of particles to which an individual has been exposed. Also, such analyses can show where potentially toxic particles accumulate. Recently, we used analytical electron microscopy to determine parenchymal particle burden in the lungs of long-term residents of Vancouver who had never smoked tobacco (*10*). Our analysis indicated that 96% of the retained particles were < 2.5 μm in aerodynamic diameter ($PM_{2.5}$), therefore suggesting that epidemiologic investigations should focus on this size class of particles.

In demonstrating biological plausibility it is important to establish a link between ambient concentrations, exposure, and dose. In this study we examined lungs from female, nonsmoking, long-term residents of Mexico City, Mexico, a region with high ambient particle levels, and Vancouver, British Columbia, Canada, a region of much lower levels. In doing so we asked a fundamental question: Does residence in a location with high air pollution levels result in a higher level of biologically delivered dose of pollutants? It is our hypothesis that exposure to high levels of particulate air pollution is reflected in increased interstitial particle burdens. Although this hypothesis may appear simplistic, there has been no direct demonstration that increased ambient particle exposure in fact results in higher particle retention (and, by implication, deposition) in the lung over a lifetime. Such a finding would provide pathologic evidence to support the epidemiologic data associating particulate matter exposure with adverse health outcomes such as mortality. This would provide additional evidence that the observed epidemiologic associations, especially those related to chronic exposures, are in fact biologically plausible. A failure to prove this hypothesis would suggest either that the observed epidemiologic associations may be driven by soluble particles (which would be cleared from the airways and parenchyma) or that the epidemiologic findings are not valid and hence argue against their plausibility.

Materials and Methods

Case selection. The study protocol was reviewed and approved by the University of British Columbia Clinical Research Ethics Board (Approval C96-0511). Lungs for this study were obtained from a general autopsy service at a cardiovascular referral hospital in Mexico City and were compared to lungs obtained from a general hospital autopsy population in Vancouver. To reduce the possibility of occupational dust exposures, only lungs from women were examined. Occupational, smoking, and residential histories were

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This work was supported by grants from the British Columbia Lung Association and the Medical Research Council of Canada. M. Brauer acknowledges the support of a Career Investigator Award from the American Lung Association and a Scientist Award from the Medical Research Council of Canada and the British Columbia Lung Association.

Received 17 January 2001; accepted 4 April 2001.

obtained by interviews with relatives using a standardized questionnaire. All subjects were lifetime nonsmokers, and none had known occupational dust exposure, including, for the Mexico City lungs, domestic wood smoke exposure. Exposure to environmental tobacco smoke was assessed by evaluation of calcium particles in tissue samples. Retained calcium particles indicate exposure to tobacco smoke (*11*). The lungs from Mexico were collected from women who had been lifetime residents of Mexico City, and the lungs from Vancouver were from residents who had lived in Vancouver for ≥ 20 years. In both locations, inclusion criteria were restricted to cases > 60 years old at time of death. The mean ages were 67 ± 19 (SD) and 64 ± 9 years for Vancouver and Mexico City, respectively. None of the patients had died of lung disease, and the lungs were all morphologically normal except for the presence of minor degrees of pneumonia at autopsy. Four additional cases from Mexico City were examined, but three were excluded because particle levels in the samples were too high to allow for quantitative electron microscopy analysis. An additional sample from Mexico City was excluded because approximately 30% of the particles were determined to contain calcium, an indicator for tobacco smoke exposure (*11*). The total number of retained particles for this case was similar to the other cases from Mexico City. Four additional cases from Vancouver were analyzed, but were excluded from the data analysis because interviews could not be conducted; consequently occupational histories were not obtained. For three of these cases, retained particle concentrations were similar to the other cases from Vancouver, whereas the concentrations from the fourth case, which appeared to be an outlier, were significantly higher.

Tissue dissection and particle counting procedure. All tissues were handled with dust-free gloves. Dissections were performed on formalin-fixed lungs using a dissecting microscope. From each specimen, we selected for analysis a sample of parenchyma weighing 1–2 g from the central portion of the lung, avoiding large airways, and an equivalent size sample that was dried to constant weight to allow expression of results as particles per gram dried tissue. We selected the central tissue sample so that we would analyze comparable tissues from Vancouver and Mexico City cases. Tissue samples were dissolved in bleach and centrifuged at 30,000 × *g* for 20 min; the sediment was washed once to remove the bleach and recentrifuged at 30,000 × *g* for 20 min to ensure that very small particles were not lost during preparation. The preparation was resuspended and collected on 0.1µm filters (Millipore-MF; Millipore Corp., Bedford, MA, USA) and then transferred to coated electron microscope grids (*10*). We previously showed that this approach effectively collects particles of ≥ 0.010 µm (*12*).

For this study, particles larger than 0.010 µm were counted, sized, and identified using an electron microscope (Phillips 400T; Phillips Electronics, Alomelo, The Netherlands) equipped with an energy dispersive X-ray spectrometer (Kevex; Thermo-Kevex X-Ray, Scotts Valley, CA, USA). Approximately 100 particles were counted per sample; particles were measured and identified by a combination of morphology and chemistry as determined by X-ray spectroscopy. For this study particles were characterized as silica, silicates, singlet particles of metals (particles analyzing only as iron,

Table 1. Concentrations of particles (millions of particles per gram of dry tissue) of different types counted in individual samples of lungs from Vancouver residents.

Sample	Silica	Silicate	Metals (single particles)	Carbon Agg	Carbon + sulfur Agg	Kaolin-like Agg	Iron Agg	Misc
42318	67	280	75	ND	ND	ND	ND	8
42313	9	40	16	ND	ND	ND	ND	ND
42324	81	119	46	ND	ND	ND	ND	ND
42304	60	143	56	ND	ND	ND	ND	ND
42329	145	220	95	ND	ND	ND	ND	ND
2458	307	119	72	ND	ND	ND	ND	ND
2459	249	49	16	ND	ND	ND	ND	ND
2460	325	150	40	ND	ND	ND	ND	ND
2461	56	71	62	ND	2	ND	ND	ND
2464	105	139	84	ND	ND	ND	ND	ND
2467	66	88	35	ND	ND	ND	ND	ND
Mean	133	128	54	0	0.2	0	0	0.7
SD	109	7	26	0	0.6	0	0	2.4
Percent of total[a]	37.9	43.0	18.8	0.0	1.0	0.0	0.0	1.9

Abbreviations: Carbon Agg, aggregated particles producing no X-ray peak; Carbon + Sulfur Agg, aggregated particles producing only a sulfur X-ray peak; Iron Agg, aggregated particles analyzing as iron, sometimes with a small silicon peak; Kaolin-like Agg, aggregated particles with a composition similar to kaolinite; Misc, miscellaneous; ND, not detected.
[a]Mean percentage of each type of particle relative to the total number of all types of particles for each case.

Table 2. Concentrations of particles (millions of particles/g dry tissue) of different types counted in individual samples of lungs from Mexico City residents.

Sample	Silica	Silicate	Metals (single particles)	Carbon Agg	Carbon + sulfur Agg	Kaolin-like Agg	Iron Agg	Misc
2416	128	132	48	48	ND	135	10	16
2417	252	1,619	352	100	100	353	ND	ND
2418	217	1,026	116	ND	16	150	251	17
2419	366	230	107	53	32	97	ND	53
2420	192	187	42	16	16	11	37	ND
2423	316	185	86	95	23	24	ND	23
2425	7,262	11,923	2,604	3,776	ND	871	ND	ND
2426	173	236	79	165	52	43	ND	25
2427	770	1,057	258	542	171	199	ND	ND
2428	3,395	8,068	4,243	3,820	1,697	848	ND	212
2448	319	1,033	73	344	25	442	ND	24
Mean	1,217	3,915	1,384	1,537	549	312	132	71
SD	2,215	2,336	728	895	236	288	99	52
Percent of total	24.6	38.2	10.1	10.9	3.2	9.3	2.1	1.6

Abbreviations: Agg, aggregated particles; Misc, miscellaneous; ND, not detected.
[a]Mean percentage of each type of particle relative to the total number of all types of particles for each case.

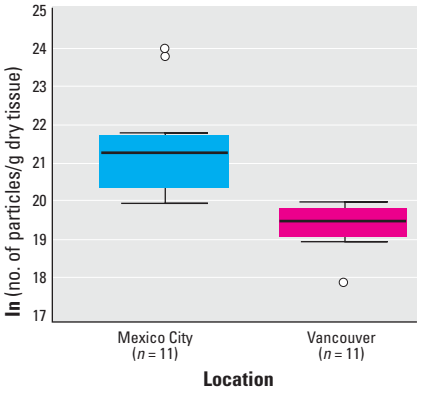

Figure 1. (Ln)Concentration of total particles per gram of dry tissue in Mexico City and Vancouver samples. The top and bottom of boxes indicate the 25th and 75th percentiles, respectively, and the length of boxes is interquartile distance. Upper and lower whiskers extend to the largest and smallest measured values that are 1 interquartile distance from the 75th and 25th percentiles, respectively. Circles are data points that are greater or less than 1 interquartile distance from the 75th or 25th percentiles. The line inside the box indicates the median value.

aluminum, or titanium), and aggregated particles (Tables 1 and 2). With one exception, the aggregated particles were only seen in Mexico City lungs. We classified aggregated particles as follows: *a*) purely carbonaceous if they were composed of more or less spherical particles that produced no X-ray signal [we previously demonstrated our ability to detect purely carbonaceous aggregates by carrying a sample of pure ultrafine carbon black through our preparative procedure, including adding a sample to lung tissue (*12*)]; *b*) carbonaceous + sulfur if they had a similar morphologic appearance but produced a small sulfur peak; *c*) kaolinite-like if they were composed of platy particles with an aluminum:silicon ratio similar to kaolin; and *d*) iron aggregates if they produced X-ray peaks for iron or iron with a small amount of silicon. For purposes of calculating particle numbers and sizes, we treated each aggregate as one particle, but we made additional measurements to determine the sizes of particles that made up the carbonaceous and carbon + sulfur aggregates. Retained particle concentrations were not normally distributed and were therefore log-transformed before all statistical analyses.

Ambient air samples. A limited number of ambient $PM_{2.5}$ particle samples were collected on filters in Mexico City and Vancouver. The purpose of this sampling was to establish whether the types of particles observed in tissue samples were of similar composition and morphology to those found in ambient air. All particle samples were collected by intermittent sampling (1 min of sampling in each 8-min period, for a total of 1,440 min) over a 7-day period in order to provide a sample that was representative of typical particle types. In both locations, samples were collected between October 1999 and January 2000. Particles were collected with Harvard Impactors on polytetrafluoroethylene (Teflon) membrane (Teflo; Pall Life Sciences, Ann Arbor, MI, USA) filters at a flow rate of 4 L/min. In Vancouver, samples were collected at a National Air Pollution Surveillance monitoring site (Kitsilano), and in Mexico City, samples were collected at two sites that are part of the Mexico City ambient monitoring network: one located in the center of the city (Hangares) and another in the southwest (Tlalpan). Three-year average PM_{10} concentrations were 66 μg/m^3 for seven monitoring sites in Mexico City and 14 μg/m^3 from nine sites in Vancouver (*13*).

After sample collection, filters were weighed and then processed for electron microscopy. The filters were wet with 0.1 mL of 95% ethanol, sonicated in 1 mL of distilled, deionized water, centrifuged, and transferred to electron microscope grids following the same procedures used for the tissue samples.

Results

We found significantly higher ($p < 0.001$, *t*-test) concentrations of retained particles in tissue samples from Mexico City than in those from Vancouver (Figure 1, Tables 1 and 2). The geometric mean total particle concentrations in the Mexico City lungs was $2{,}055 \times 10^6$ particles/g dry lung [geometric SD (GSD) = 3.9] as compared to 279 (GSD = 1.8) $\times 10^6$ particles/g dry lung in the Vancouver samples, a nearly 10-fold difference. Examination of individual mineral species showed higher particle concentrations in the Mexico City samples for every particle type examined (compare mean concentrations in Tables 1 and 2).

In addition to the mixture of silicates and other crustal material typically found in tissue samples, the samples from Mexico City contained on average 25.5% aggregated ultrafine particles (Table 2). In particular, we observed chain aggregates of approximately spherical particles that produced no energy dispersive X-ray signal and were, therefore, presumably carbonaceous (Figure 2). Many of these also contained trace amounts of sulfur, which is suggestive of combustion source particles. The morphology of the chain aggregates was remarkably similar to those isolated from Mexico City ambient air samples (Figure 2A) and from diesel exhaust (*14*). In sharp contrast to the Mexico City samples, only 1 aggregate (carbonaceous + sulfur) was detected in the 11 Vancouver tissue samples (Table 1). In Mexico City tissue samples, a large number of aluminum silicate aggregates with a chemical composition similar to kaolinite were also identified, as were occasional aggregates consisting of iron particles that also gave a small X-ray peak for silicon. The origin of these particles was unclear, but they were never observed in Vancouver lungs. On average, the aggregated carbonaceous particles and carbonaceous particles + sulfur made up 14% of the total particles; the kaolinite-like aggregates made up 9%, and the iron aggregates 2% (Table 2). However, if every particle in the aggregates was counted as a single particle, these particles would make up the vast majority of the particles detected in the Mexico City tissue samples.

Tables 3 and 4 show the sizes of particles in the lung tissue samples from the two sites. Overall, the geometric mean particle size in the lungs was similar in both cities, with a mean for all of the cases of 0.35 μm for Mexico City samples and 0.39 μm for Vancouver samples. Table 4 also shows the geometric mean diameters for the aggregated particles detected in lungs from Mexico City. Some of the aggregates were quite large, ranging up to about 4 μm, but most were smaller than 1 μm. Table 5 shows the mean sizes of the particles that made up the carbonaceous and carbon + sulfur aggregates. These were almost all ultrafine particles. The structure of the kaolinite-like aggregates and iron aggregates prevented measurement of individual particle sizes.

Comparison of air samples from the two locations indicated a similar distinction in overall mass (and particle number) concentrations and in composition, with more than 20 times as many aggregates observed in

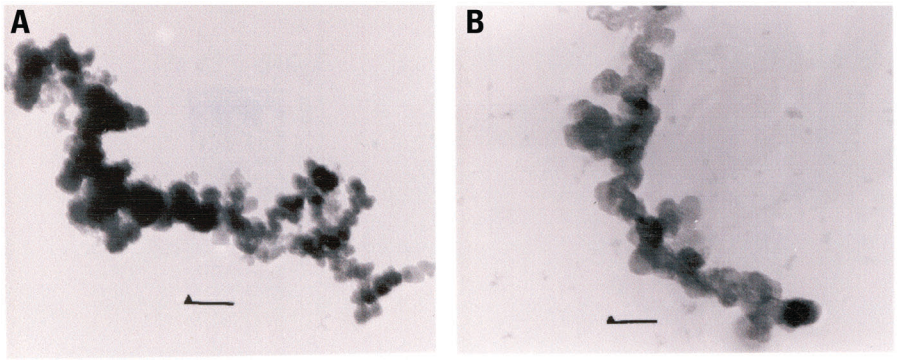

Figure 2. Representative illustration of chained aggregated spherical particles giving no signal (i.e., carbonaceous particles) from (*A*) a Mexico City air sample and (*B*) a Mexico City lung. Bars = 0.1 μm.

Table 3. Geometric mean (GSD) particle diameters (μm) for individual samples of lungs from Vancouver.

Sample	All particles	Carbon + sulfur Agg
42318	0.69 (2.3)	ND
42313	0.69 (2.2)	ND
42324	0.52 (2.2)	ND
42329	0.65 (2.5)	ND
2458	0.31 (2.7)	ND
2459	0.22 (2.3)	ND
2460	0.33 (2.4)	ND
2461	0.31 (2.6)	0.33 (0)[a]
2464	0.31 (2.3)	ND
2467	0.34 (2.3)	ND

ND, not detected. Each aggregate was counted as one particle. No carbon aggregates, kaolin-like aggregates, or iron aggregates were detected in any of the samples from Vancouver.
[a]Only one aggregate identified.

Mexico City samples than in those collected in Vancouver. A more quantitative comparison was not possible because many of the ambient samples collected in Mexico City contained too many aggregates to reliably count. For the limited samples that we collected, the mean $PM_{2.5}$ particle mass concentration measured in Mexico City was 29.5 μg/m^3 (n = 11) compared to a mean concentration of 10.5 μg/m^3 for the samples (n = 6) collected in Vancouver. The geometric mean diameter of ambient carbon aggregates (counting the entire aggregate as one particle) from Mexico City was approximately 1.1 μm, with individual particles within the aggregates in the range of 0.04– 0.15 μm. Because of their complex morphology, it was not possible to determine individual particle sizes for the kaolinite-like aggregates observed in air samples collected in Mexico City.

Discussion

Our observations indicate that long-term residence in an area of high ambient particle concentrations is associated with greater numbers of retained particles in the lung; this shows for the first time that the aggregated ultrafine particles in ambient air can also be found in lung tissue. Our ability to detect retained aggregated ultrafine particles provides evidence that aggregates in air do not disaggregate once they are inhaled, although the sizes in tissue samples were slightly smaller than in air. We cannot determine absolutely if the aggregates we observed in tissue samples are the same as those observed in air samples. However, the similarities between the two (Figure 2) make it unlikely that the aggregates observed in the lungs form after inhalation of airborne ultrafine particles or that they are artifacts of the extraction procedure.

This work, and conclusions that may be drawn from it, is subject to several limitations. In both locations, we observed a large degree of intersubject variability in numbers of retained particles (Figure 1, Tables 1 and 2). This is likely the result of variable exposures as well as interindividual differences in particle clearance and translocation efficiency. Although we have clearly found a difference in the number of retained particles between tissue samples of residents of Vancouver and Mexico City, we were unable to identify differences in the numbers of retained particles in individuals living in higher and lower pollution regions of Mexico City.

Because of the complexity of the analysis and the difficulties in obtaining autopsy samples that meet our inclusion criteria (nonsmoking women > 60 years at death, > 20 year residence in Vancouver or Mexico City, no occupational dust exposure, no deaths from respiratory disease), our sample size was limited and the measured concentrations of retained particles should not be considered quantitatively representative of those for individuals living in Vancouver or Mexico City. However, our analysis shows that the sample size was sufficient to indicate a statistically significant difference between the groups from the two locations. The exclusion of four samples from Mexico City with particle levels that were too high to allow for quantitative electron microscopy analysis does not alter this finding. Had we been able to quantify the high particle levels on these samples, the differences between the two locations would have been even greater.

Our inclusion criteria allowed us to at least partially control for confounding by sex, smoking, age, and duration of residence while we also screened samples for calcium particles as indicators of environmental tobacco smoke exposure. Although we believe that these are the major potential confounding variables of concern for this analysis, it is possible that other unrecognized factors pertaining to differences between the study populations from the two locations contributed to the observed differences.

The number of retained particles we observed is certainly a marked underestimate of the number inhaled because many particles are soluble and therefore would not be detected by our procedures. Further, our analytical approach cannot differentiate between particles originating in airspaces and those that have entered the interstitium, so that we cannot determine what proportion of measured particles have been very recently inhaled. However, our data clearly indicate that, despite exposure to similar types of particles, individuals who reside in an area of high compared to low ambient particle concentrations retain much greater numbers of ambient particles. This finding may seem trivial, but it should be considered in the context of the low mass concentrations of particles in ambient air compared to occupational dust exposures that lead to disease. This finding suggests that even the gravimetrically small particle burden found in regions with high concentrations of ambient particles is able to overwhelm local clearance mechanisms, presumably as a result of particle toxicity.

In conclusion, we observed significantly higher numbers of retained particles in lung tissue samples from long-term residents of Mexico City, a region with high ambient air pollution, relative to samples from long-term residents of Vancouver, a region with much lower ambient pollution levels. Because we restricted our analysis to tissue samples from nonsmoking women, it is likely that the differences observed were due to differences in ambient exposures. Additionally, aggregates of ultrafine particles can be found in large numbers in the lungs of individuals from Mexico City, but were only rarely observed in samples from Vancouver. These particles are morphologically and chemically similar to particles found in ambient air, and at least some of these particles appear to be combustion derived on the basis of morphologic and chemical similarities to particles from motor vehicle exhaust. Our observations demonstrate, therefore, that long-term exposure to ambient particles, and especially to aggregated ambient ultrafine combustion products, results in higher retention of these particles in lung tissue. Because the findings demonstrate

Table 4. Geometric mean (GSD) particle diameters (μm) for individual samples of lungs from Mexico City.

Sample	All particles	Carbon Agg	Carbon + sulfur Agg	Kaolin-like Agg	Iron
2416	0.47 (2.6)	0.40 (2.0)	ND	0.65 (2.3)	0.13 (1.2)
2417	0.39 (2.5)	0.56 (2.1)	0.48 (1.1)	0.52 (1.3)	ND
2418	0.23 (2.5)	ND	0.89 (1.1)	0.78 (2.1)	0.62 (1.7)
2419	0.41 (2.4)	0.44 (1.7)	2.0 (2.8)	0.61 (1.5)	ND
2420	0.37 (2.5)	0.32 (1.5)	0.43 (0)	0.64 (1.8)	0.64 (1.8)
2423	0.38 (2.7)	0.62 (1.6)	1.4 (2.2)	1.29 (1.3)	ND
2425	0.35 (2.8)	0.44 (1.3)	ND	0.38 (2.7)	ND
2426	0.29 (2.3)	0.40 (1.7)	0.48 (1.8)	0.44 (2.1)	ND
2427	0.36 (2.7)	0.30 (1.6)	0.40 (1.7)	0.67 (1.7)	ND
2428	0.25 (2.2)	0.36 (1.4)	0.35 (1.3)	0.52 (1.6)	ND
2448	0.36 (3.4)	0.44 (1.7)	0.31 (3.8)	1.28 (2.6)	ND

ND, not detected. Each aggregate was counted as one particle.

Table 5. Geometric mean (GSD) particle diameters (μm) for individual particles in aggregates in samples of lungs from Mexico City and Vancouver.

Sample	Carbon Agg	Carbon + sulfur Agg
Mexico		
2416	0.073 (1.1)	ND
2417	0.077 (3.6)	0.12 (1.0)
2418	ND	0.25 (1.0)
2419	0.073 (2.9)	0.097 (2.7)
2420	0.054 (1.0)	0.090 (2.5)
2423	0.12 (1.9)	0.17 (1.7)
2425	0.069 (1.7)	ND
2426	0.046 (2.8)	0.075 (2.1)
2427	0.049 (1.9)	0.058 (1.9)
2428	0.027 (1.3)	0.047 (1.9)
2448	0.038 (2.3)	0.019 (1.0)
Vancouver		
2461	ND	0.041 (1.0)[a]

ND, not detected.
[a]Only one aggregate identified.

a link between ambient particle concentrations and a measure of biologically relevant dose, they support the biological plausibility of adverse health effects being associated with exposure to particulate air pollution.

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Acute Sensory Responses of Nonsmokers at Very Low Environmental Tobacco Smoke Concentrations in Controlled Laboratory Settings

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The objective of this study was to provide a basis for effectively protecting nonsmokers from acute sensory impacts and for preventing deterioration of indoor air quality caused by environmental tobacco smoke (ETS) emissions. With an olfactory experiment we determined odor detection thresholds (OT) of sidestream ETS (sETS), and with a full-body exposure experiment we investigated sensory symptoms at very low sETS exposure concentrations. OT concentrations for sETS are three and more orders of magnitude lower than ETS concentrations measured in field settings and correspond to a fresh air dilution volume of > 19,000 m^3 per cigarette, over 100 times more than had previously been suggested for acceptable indoor air conditions. Eye and nasal irritations were observed at one order of magnitude lower sETS concentrations than previously reported, corresponding to a fresh air dilution volume of > 3,000 m^3 per cigarette. These findings have great practical implications for defining indoor air quality standards in indoor compartments where ETS emissions occur. Our study strongly supports the implementation and control of smoking policies such as segregating smoking areas from areas where smoking is not permitted or instituting smoking bans in public buildings. Key words: environmental tobacco smoke, indoor air quality, odor threshold, sensory symptoms, ventilation. ***Environ Health Perspect*** **109:1045–1052 (2001). [Online _____]**
http://ehpnet1.niehs.nih.gov/docs/2001/109p1045-1052junker/abstract.html

Over the past years, several studies evaluating acute health impacts and sensory responses from exposure to environmental tobacco smoke (ETS) have been performed. Chamber studies, evaluating lung functions of asthmatics and other sensitive subjects, have used sidestream ETS (sETS) concentrations between 2 and > 15 ppm carbon monoxide (*1–3*), and studies focusing on sensory symptoms have used ETS at lower concentrations (*4–7*). For eye irritations, a tolerable limit of 1.5–2 ppm CO has been reported (*5–8*). Significant increases of perceptive eye and nasal irritations as well as annoyance were observed at respirable suspended particulate matter (RSP) concentrations of 58 µg/m^3, corresponding to a time-weighted average concentration of 0.22 ppm CO, and led to a significant decrease in air quality acceptability (*7*). The authors estimated that an 80% air quality acceptability rate corresponded to an RSP concentration of 103.3 µg/m^3. Based on an average ETS-RSP yield per cigarette of 13.7 mg (*9*), this concentration corresponds to one cigarette diluted in an average western European living room. Cain et al. (*4*) reported similar observations.

Regarding the typical exposure concentrations encountered in field studies, RSP concentrations are reported at 120 µg/m^3 when someone is smoking (*10*). More recent personal exposure studies in the United States and in Europe showed median RSP concentrations that were markedly lower (*11–14*). However, these data are based on sample intervals averaged over 8-hr periods. Short-term RSP concentrations have been reported to be much higher (*10,15*). Furthermore, an alarming increase in the active smoking rate has been observed in some countries. In Switzerland, an increase of greater than 40% has been reported in the 14–24 years age group (*16*).

The awareness that perceptual and comfort aspects are important factors in a healthy building is growing, and indoor air quality guidelines are taking this more and more into consideration (*17*). ETS, as a contributor to sick building syndrome (*18*), potentially causes widespread sensory impacts and discomfort in many places where smokers and nonsmokers coexist. This concept is supported by the observation that people with a history of atopy or respiratory illness are more sensitive to the acute, irritating effects of ETS than people without such a medical history (*19*). However, odor thresholds and thresholds of perceptive irritations with respect to ETS have not been determined conclusively. The World Health Organization recommends that unwanted odorous compounds should not be present in concentrations exceeding the ED_{50} (effective dose that makes 50% of the exposed population respond) detection threshold. Sensory irritants should not be present in excess of the ED_{10} (effective dose that makes 10% of the exposed population respond) detection threshold (*20*). That many public buildings, schools, and restaurants still do not implement smoking policies in several parts of the world today indicates that ETS is potentially present and constitutes a social problem now and in the future.

The goal of this study was to determine odor detection thresholds of sETS in a laboratory setting. Acute sensory symptoms, breathing patterns, annoyance, and the indoor air quality acceptability were determined at very low sETS concentrations in an exposure chamber. On the basis of sETS emission rates, we established fresh air volumes necessary to dilute one cigarette to threshold concentrations. In addition, we used startle reflexes that are assessed by electromyogram recordings of the M. orbicularis oculi and elicited by an acoustic stimulus as an objective indicator of annoyance.

In this study, we aimed to determine air quality standards required to protect nonsmokers from adverse health effects caused by impacts of ETS on the human sensory system as well as to provide measures for establishing acceptable indoor air quality. We show that ETS odor thresholds are about 100 times lower, and nasal and eye irritations about 10 times lower, than reported in previous studies (*4,7*). On a practical level, separately ventilated areas for smokers and nonsmokers or a complete smoking ban are required to protect nonsmokers effectively from the sensory impacts and the annoyance potential of ETS.

Methods

Experimental design. In this study, we performed three experimental sessions. During one session, we conducted an olfactory experiment determining sETS odor detection thresholds. Data obtained laid the foundation of a laboratory exposure study investigating

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We thank H.C. Siegmann and his team from the Laboratory for Solid State Physics for their excellent collaboration. Among them great appreciation goes to P. Cohn for his invaluable technical support. We thank M. Hangartner for the use of the olfactometer, S.I. Chol for technical assistance, S. Junker for laboratory assistance, and R. Knutti and N. Achermann for analyzing the VOCs. We also thank T. Blumenthal and R. Waeber for their excellent input.

Received 30 May 2000; accepted 21 March 2001.

sensory symptoms in nonsmokers at very low sETS concentrations. Figure 1 illustrates the basic design scheme of the experimental setup for both studies. Moreover, we performed a cigarette emission study in the empty exposure chamber to describe the results obtained in terms of cigarette equivalents. We could thus compare sETS generated for both the exposure and olfactory study to sETS not biased by the experimental setup.

We generated sETS in a glove box 0.6 m^3 in volume by a Borgwaldt smoke generator (Borgwaldt, Hamburg, Germany). On the basis of sales statistics of the Swiss Community of the Cigarette Industry, we chose six cigarette brands and evenly distributed them on the smoke generator (*21*). Throughout the duration of a session, two randomly chosen cigarettes burned until they passively extinguished after 5–6 min. When burning ceased, another two cigarettes were lit. The mainstream fraction of the tobacco smoke aerosol was exhausted out of the glove box into a ventilation hood.

Fresh air was introduced into either the olfactometer or the full-body exposure chamber by a fresh air unit, equipped with two radial ventilators providing a fresh air flow of 1.5 m^3/min. The air was filtered by a glass fiber filter (Camfil 1E-110; Camfil AB, Trosa, Sweden) and an active charcoal granulate (CN-50 6 × 12 1.7–3.4 mm; Siegfried AG, Zofingen, Switzerland).

Cigarette emission experiment. To establish the amount of sETS emitted by one cigarette, we multiplied average baseline-corrected ETS concentrations throughout the burning time of the cigarette (570 sec) by the amount of fresh air introduced into the empty exposure chamber during the same time period (25.7 L/sec). During the cigarette emission experiment, one cigarette of the most commonly smoked brand in Switzerland was lit and inserted through the ceiling into the empty exposure chamber 2 m^3 in volume via a PVC tube. The experiment was repeated six times. Because the cigarettes smoldered passively, they remained burning for 9.5 min. During this time no mainstream smoke was generated (i.e., no puffs were taken). Because of the rather high air exchange rates (45/hr), we assumed a homogenous distribution of sETS. The cigarettes remained burning until they passively extinguished.

Subjects. We chose 24 female nonsmokers to participate in the olfactory and the full-body exposure experiments. Written consent was obtained from the subjects before the experiments. The Ethics Commission of the Federal Institute of Technology (Zurich, Switzerland) approved the study.

The subjects were required to be healthy, not to have a record of allergy to pollen or dust, not to be anosmatic, and not to have smoked in the last 5 years. Moreover, the subjects were not permitted to use either eyeglasses or contact lenses and were asked to refrain from being exposed to ETS on the day of the study. The subjects were between 18 and 35 years of age and were paid for their participation. Of the 24 who participated in the full-body exposure study, 18 took part in the olfactory experiment. In a preliminary questionnaire, the participants were asked to indicate their degree of annoyance by ETS, automobile exhaust fumes, solvents, and perfumes.

Olfactory experiment. To obtain sETS odor thresholds, we performed two types of experiments based on the method of limits (*22*). In one, the subjects were asked to evaluate the air by placing their nose into the duct of the olfactometer only upon presentation of the stimuli (type A); in the other, the subjects' noses remained within the duct throughout the duration of the experiment (type B). In four to eight repetitions, stimuli were presented in ascending concentrations for both experiments. A potential odor threshold value within a trial was obtained when the subject perceived the ascending concentration of stimuli for the first time. A valid odor threshold value was given when a subject stated perceiving an odor during two consecutively ascending concentrations. We calculated odor thresholds by subtracting the sETS baseline concentration before the stimuli had been presented from the maximum concentration of the sETS indicator during stimuli presentation. The data were obtained from 18 female nonsmokers who were divided into six panels of three subjects per panel.

An olfactometer developed at the Institute for Hygiene and Applied Physiology (Zurich, Switzerland) was used (*23*). Air is drawn via a Teflon-coated ventilator from the surrounding environment and guided through a system of glass tubing to four Teflon-coated nose ducts. Fresh air is constantly washed through the system at a rate of 147 L/min, reaching an air speed of 0.85 m/sec at the ducts from where the sensory measurements are carried out. One of the four nose ducts was used for monitoring ETS indicators. We fed sETS manually into the fresh air stream by rotameters. The maximum dilution factor of the olfactometer is 39,400. This was doubled with a further dilution before entering the olfactometer by a factor of two.

Full-body exposure experiment. The experimental procedure performed for each participant within the exposure chamber is described qualitatively in Figure 2.

Each session consisted of eight conditions of interest. In four of the eight episodes, different amounts of sETS, distinguished by the air flow rates of 200 mL/min, 500 mL/min, 1,200 mL/min, or 3,600 mL/min, were fed from the glove box into the fresh air stream passing though the exposure chamber (sETS condition). The smallest flow rate was determined to generate sETS concentrations that were approximately equivalent to concentrations observed at the 95th percentile of the odor threshold. Before each of these sETS conditions, air without sETS (zero condition) was administered. We randomized the sequence of sETS conditions over 24 subjects. For each subject the administered ETS episode pattern was

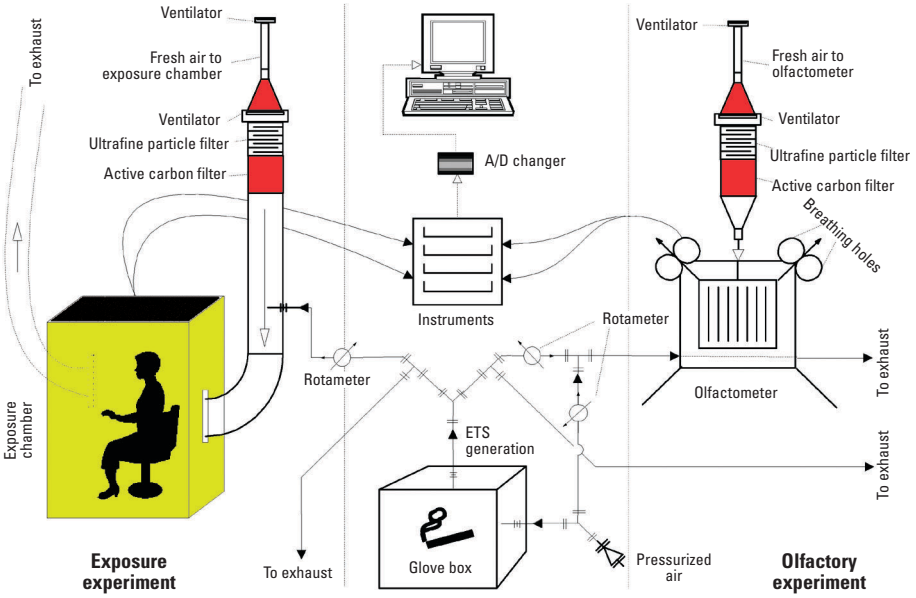

Figure 1. Scheme of the experimental setup for the odor threshold and the full-body exposure study. The equipment shown in the middle panel was used for both setups. Generated in the glove box and diluted by a fresh air delivery system, sETS was fed into either the olfactometer to determine odor detection thresholds or into the exposure chamber to assess sensory symptoms.

randomly selected out of a pool of 24 possible patterns. The session commenced with a zero condition that was succeeded by a randomly selected sETS condition. Zero condition and sETS condition then followed in alternating order. Each episode commenced with a 2-min time span of startle stimuli that was followed by a questionnaire and proceeded by an eye blink count. We continuously monitored breathing patterns throughout the session. To minimize distractions, a beige cotton curtain surrounded the exposure chamber. The experimenter did not have any eye contact with the subject.

For the sensory questionnaires, each sensory symptom was scaled on a vertical axis within which the participants were told to mark a horizontal reference anywhere on the scale that reflected their perception of the given symptom (Table 1)

The exposure chamber was constructed out of Plexiglas (height, 1.6 m; length, 1.4 m; width, 0.9 m). It was possible to seat a subject comfortably in front of a small desk. The fresh air unit providing particle free air at a constant volatile organic compound (VOC) background concentration maintained a constant air flow (1.5 m^3/min). Air was fed into the chamber via a ventilation duct (0.25 m in diameter) situated knee height near the far corner of the chamber on the right hand side facing the participant. The exhaust air left the chamber by a duct (0.25 m in diameter) behind the subject's head. In this way the air was forced to pass by the subject's face. Although the air exchange rate of the ventilation system was 45/hr, air velocities in the vicinity of the face remained < 0.1 m/sec. Air sampling tubes were placed through holes in the center part of the ceiling near head height.

Instrumentation. A number of sETS constituents were continuously monitored throughout the duration of the experiments:

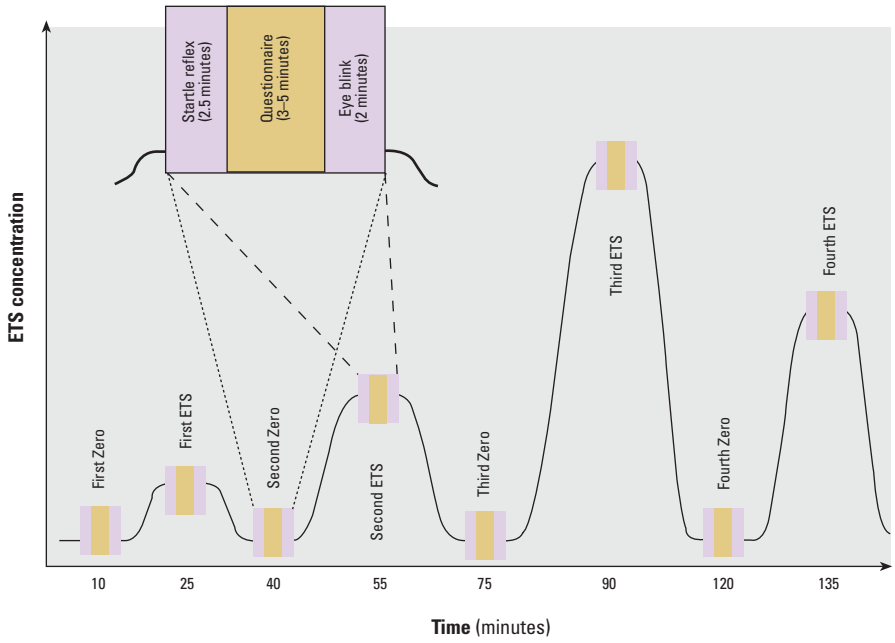

Figure 2. Experimental procedure during one session of the exposure experiment. The sequence of sETS concentrations was randomized over 24 subjects. Each episode commenced with a startle response measurement, followed by a questionnaire and an eye blink count. Breathing pattern measurements were performed during the entire session.

Table 1. A streamlined version of the sensory symptoms questionnaire.

Assessed judgment	Scale
Air temperature Relative humidity	3, too high; 0, just right; –3, too low
Odor strength Eye irritation Nasal irritation Throat irritation	6, overwhelming; 5, very strong; 4, strong; 3, moderate; 2, weak; 1, very weak; 0, not at all
Arousal Annoyance	6, overwhelming; 5, very strong; 4, strong; 3, moderate; 2, weak; 1, very weak; 0, not at all
Odor perception	1, extremely pleasant; 0.67, pleasant; 0.33, rather pleasant; 0, neutral; –0.33, rather unpleasant; –0.67, unpleasant; –1, extremely unpleasant
Odor perception, air quality	1, acceptable; –1, unacceptable; a value > 0 is acceptable; a value < 0 is unacceptable

particle-bound polycyclic aromatic hydrocarbons (pPAH), total volatile organic compounds (tVOC), and particle number concentrations. In the cigarette emission and full-body exposure experiment, CO was additionally monitored, and a number of discrete particle number and particle mass distributions were carried out. CO_2, parameters of thermal comfort, and VOCs were also assessed in the full-body exposure study.

We measured pPAH by means of a photoelectric aerosol sensor (PAS, type: LQ1-TV, Matter Engineering Inc., Wohlen, Switzerland) (*24,25*) For total volatile organic compounds a flame ionization detector was used (Model VE7; J.U.M. Engineering, Karlsfeld, Germany). We measured CO with an APMA-300E CO Monitor (Horiba Ltd., Japan). To assess the total particle number concentrations, we used a condensation nucleus counter (version 3025; TSI, St. Paul, MN, USA). For particle number versus size distributions, we used a scanning mobility particle sizer (version 2.3; TSI Inc.) in the size range between 0.015 and 0.673 µm mobility diameter at a resolution of 64 channels per decade on a logarithmic diameter axis. A 10-stage Quartz Crystal Microbalance Cascade Impactor System allowed the assessment of size-specific particle masses (Model PC-2; California Measurements Inc., Sierra Madre, CA, USA) at a mid-point aerodynamic cutoff ranging from 0.07 µm to 35 µm.

For the chemical analysis of the VOC samples, a known volume of air was pumped through a stainless-steel tube filled with an adsorbent (Tenax TA; Tenax GmbH, Düsseldorf, Germany). The transfer of the sample to capillary gas chromatography (column: DB-5ms, 30 m; J&W Scientific, Agilent Technologies, Palo Alto, CA, USA) was done by thermodesorption (Perkin Elmer ATD 400; Perkin Elmer Instruments, Wellesley, MA, USA). The gas chromatrgraph (Fisons 6000; Fisons Instruments, Beverly, MA, USA) was equipped with an flame ionization detector for quantification and a mass spectrometer (Fisons MD800) for identification of the detected VOCs. The sorbent tubes were loaded with toluene-d8 as an internal standard. Concentrations are given as toluene equivalents. The Tenax tubes were exposed for 60 min at a sample rate of 100 mL/min. The sampling and analysis of these VOCs was performed by the Swiss Federal Department for Economics and Occupation (Zurich, Switzerland). For the aldehyde analysis, samples were drawn through a stainless-steel tube at a sampling rate of 1.3 L/min with 2,4-dinitrophenylhydrazine as an adsorbent. Aldehydes are desorbed, and via high pressure liquid chromatography the different species are determined. The aldehyde analysis was performed by the Institut

für Gefahrstoff-Forschung der Bergbau-Berufsgenossenschaft (Bochum, Germany). The data for both the VOCs and the aldehydes are not shown.

We measured the air temperature and relative humidity with an instrument from ROTRONIC AG (Bassersdorf, Switzerland). Wind speeds were assessed by a Dantec low velocity flow analyzer type 54N50 (Dantec Inc., Copenhagen, Denmark). Carbon dioxide measurements were performed with the EGQ-10 measuring instrument (Sauter AG, Basel, Switzerland).

We recorded respiratory parameters by Respitrace cardio respiratory diagnostic technology (SensorMedics Technology, Yorba Linda, CA, USA) based on inductive plethysmography. Data analysis was performed with RespiEvents software (version 4.2c; Nims, Miami Beach, FL, USA). Breathing bands that assessed breathing patterns were fitted over the subject's breasts and abdomen. We calibrated the bands before and after the experiment using a spirometer (Spiro-Junior; Erich Jaeger, Würzburg, Germany).

We used an SR-EMG System (San Diego Instruments Inc., San Diego, CA, USA) to assess the startle response signal. This device is a modularized electromyograhic system of two units, an amplifier modifier and a stimulus generator unit. For the startle response measurements, we placed two electrodes on the M. orbicularis oculi of the left eye of the subject. A broad-band white noise (100–1,000 Hz) at 65 dB_A as a background was presented to the subject during a 2-min period over a set of headphones. During this period a series of 10 acoustic impulses of 100 dB_A for a time span of 40 msec were generated.

Results

Cigarette Emission Experiment

The emission rates per cigarette for pPAH, $PM_{2.25}$, particle numbers, CO, and tVOC are shown in Table 2.

To estimate the degree that coagulation and adsorption processes may alter the physical characteristics of the sETS aerosol, we compared particle number and particle mass distribution measurements from directly emitted sETS to machine-generated sETS that had been transferred from the glove box to the exposure chamber. The particle number distribution of one cigarette burning in the exposure chamber shifted from a geometric mean diameter of 0.085 µm (geometric standard deviation = 0.002 µm) to an average geometric mean diameter of 0.172 µm (geometric standard deviation = 0.002 µm) when initially generated in the glove box (average of 3 measurements). Parallel to the increase in mean diameter, the particle number concentration would have to decrease over time. Based on the particle emission rate of 9.3×10^{12} particles per cigarette (Table 2), the estimated particle concentration in the glove box (0.6 m^3) after two cigarettes had burned was 3.1×10^7 particles/cm^3. The following calculations were performed to estimate the actual particle number concentrations if coagulation processes in the glove box had not taken place (*26*):

$$N(t) = \frac{N_0}{1 + N_0 K t} \quad [1]$$

$$K = C_c 3.0 \times 10^{-10} \quad [2]$$

$$\frac{d(t)}{d_0} = \left(\frac{N_0}{N(t)}\right)^{1/3} \quad [3]$$

where $N(t)$ = particle number concentration at time t; N_0 = initial particle number concentration = 3.1×10^7; K = coagulation coefficient; C_c = slip correction factor ~ 1.2 for a particle with a geometric mean diameter of 0.085 µm; t = approximate burning time of a cigarette including time to transfer to the exposure chamber, ~ 420 sec; $d(t)$ = particle diameter at time t; and d_0 = initial particle diameter = 0.085 µm.

The solution to Equation 1 equals 5.5×10^6 particles/cm^3 (i.e., 5.7 greater particle numbers if coagulation had not taken place), and the geometric mean diameter increased by a factor of 1.78 (Equation 3). Compared to the initial particle number concentration, this is equivalent to a theoretical decrease by a factor of 5.7 after coagulation in the glove box and adsorption of the smaller particles onto the PVC tubes has taken place. The observed increase in geometric mean diameter by a factor of 2.02 is similar to the calculated increase of 1.78. In addition, the particle mass distribution revealed a shift to larger diameters within the accumulation mode (0.1–2 µm) after sETS had been generated in the glove box and transferred to the exposure chamber (data not shown). These results show that substantial coagulation and particle removal have taken place in the time span between aerosol generation within the glove box and its analysis in the exposure chamber.

Table 2. Average sETS emission rates per cigarette.

Indicators	Mean concentration during burning time ± SD	Air volume during burning time of one cigarettes (m^3)	sETS generation per cigarette ± SD
pPAH	1,661 ± 117 ng/m^3	14.65	24.3 ± 1.7 µg
$PM_{2.25}$[a]	387 ± 78 µg/m^3	14.65	5.7 ± 1.1 mg
Particle numbers[b]	(6.3 ± 0.5) × 10^5/cm^3	14.65	(9.3 ± 0.7) × 10^{12}
CO	4.88 ± 0.47 ppm	14.65	89 ± 9 mg
tVOC[c]	3,722 ± 414 ppb	14.65	113 ± 13 mg

The experiment was repeated six times.
[a]One profile was generated. [b]Particle numbers were averaged out of three repetitions. [c]tVOC masses were calculated on the basis of propane equivalents.

Olfactory Experiment

The obtained odor thresholds of sETS expressed in terms of measured particle numbers, pPAH, and tVOC concentrations are depicted in Figure 3.

The comparison of both experiment types shows an increase in sensitivity of the odor threshold based on median sETS concentrations by a factor of 2–4 while the subjects' noses remained in the ducts. The variability of all measurements expressed by the ratio between the 95th and 5th percentile lies between 9 and 35 (type A) and between 6 and 21 (type B). The variability based on the ratios between maximum and minimum odor threshold concentration do not exceed 300 for type A, while for type B a maximum ratio of 175 was observed.

Subjects

We chose 24 healthy, female nonsmokers for the full-body exposure study assessing a variety of sensory symptoms, startle responses, and breathing patterns in a range of very low ETS concentrations. Before the study the participants were asked to state how bothered they

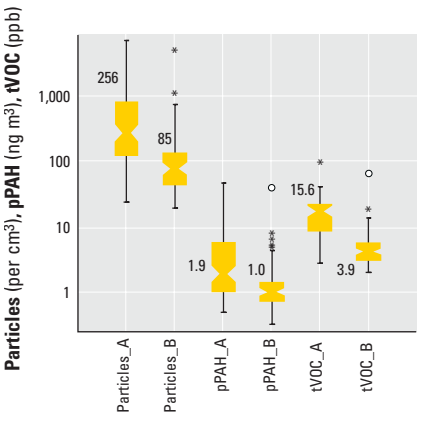

Figure 3. Odor detection thresholds of sETS expressed in terms of particles per cubic centimenter (number of values: 98 for type A, 60 for type B), pPAH, in nanograms per cubic meter (number of values: 98 for type A, 74 for type B), and tVOC, in parts per billion (number of values: 51 for type A, 75 for type B) concentrations. Two experiment types were performed: type A, nose in (5 sec) and out (30 sec) of duct; type B, nose stays in duct. Box plots were generated with Systat 8.0. Number in boxes are median concentrations.

generally felt toward ETS, automobile exhaust fumes, perfumes, and solvents. On a voting scale from 1 to 5 (1 = not at all bothered; 5 = very bothered), the subjects were, on average, more bothered by ETS (4.3) and automobile exhaust fumes (3.9) than by perfumes (2.2) and solvents (2.6). None of the subjects was very bothered by all of these agents.

Full-Body Exposure Experiment

The following sections describe the environmental conditions and the chemical species the subjects were exposed to. The results of the sensory symptom questionnaire, breathing patterns, eye blink rates and startle reflex measurements are presented.

Environmental conditions. Table 3 summarizes the average ETS concentrations of the four ETS conditions the subjects were exposed to. The ETS conditions were randomly distributed in an odd succession. Also shown are four alternating zero conditions.

The tVOC concentrations during the zero-air condition represent values that can cause possible discomfort and irritation according to the guidelines suggested by Mølhave (*27*). This remains unexplained, because the subsequent VOC concentrations measured by Tenax tubes with a sampling time of 60 min were not above the limit of detection (data not shown). The study population perceived the odor as neutral (neither pleasant nor unpleasant), and judged the quality of indoor air as acceptable.

The concentrations of VOCs and aldehydes to which the subjects were exposed increased with the degree of sETS infiltration into the exposure chamber. The data (not shown) suggest that for nicotine and 3-ethenyl-pyridine the surfaces of the glove box and the tubing acted as a sink.

Sensory responses. On the basis of the questionnaire results, we compared the average absolute sensory symptom values during the four ETS conditions to the sensory symptom values of the directly preceding zero-air condition (Table 4; only lowest sETS condition shown). The differences between the intensity of a sensory symptom at an ETS condition and symptom intensity of the preceding zero condition were statistically significant for all perceived sensory symptoms except perceived air temperature and relative humidity. The average concentrations at the lowest sETS were 468 particles per cm^3, 7.3 ng/m^3 pPAH, and 19 ppb tVOC. This corresponds to an estimated ETS-$PM_{2.25}$ (particulate matter ≤ 2.25 µm diameter) concentration of about 4.4 µg/m^3. At these concentrations the percentage of occupants judging the quality of air to be acceptable was 33%.

The results for the sensory symptoms show that even at very low ETS concentrations, subjects perceived a significant increase in sensory impact (eye, nasal, and throat irritations). Furthermore, they felt significantly more annoyed and reported the quality of air to be less acceptable than under zero conditions.

Humans are capable of discriminating relative changes only in perception (*28*). Figure 4 takes this circumstance into account. Plotted are relative increases of the intensity of a sensory symptom (intensity at an ETS condition minus intensity at the preceding zero condition) against relative increases of log-transformed sETS concentrations (ETS concentration at an ETS condition minus ETS concentration at its preceding zero condition). Furthermore, *p*-values of a linear regression model are depicted.

Based on a Pearson's linear regression model, the log-transformed ETS indicators such as particle numbers, pPAH, and tVOC concentrations show a linear trend with odor strength, eye irritation, arousal, annoyance, odor perception, acceptability of indoor air quality, wanting to open the window, wanting to leave the room, and complaining at work. Nasal irritations, on the other hand, show a linear trend with the particulate indicators only.

To determine which sensory channel (odor, nose, eyes, throat, arousal) contributes most to the observed decline of the indoor air quality, we performed a stepwise multiple linear regression model. Only the linear combination of the variables odor strength ($F = 12.1$, $p = 0.001$) and arousal ($F = 7.39$, $p = 0.008$) related to the degree of indoor air quality acceptability ($r^2 = 0.5$, $p < 0.001$). The contributions of eye, nasal, and throat irritations, however, did not show a significant relation ($p > 0.4$).

Startle reflex measurements. In the past, the startle reflex has been used as a tool to evaluate emotional qualities of a foreground stimulation (*29,30*). Because the startle reflex is not confounded by voluntary muscle activity, it is well suited to assessing motor behavior caused by a foreground stimulus. The startle reflex amplitude is affected by the extent to which the foreground stimulus can attract attention (*31*), especially when foreground stimuli and startle stimuli constitute different modalities (*32*). The startle response is facilitated when attention is directed to an acoustic startle stimulus, whereas the response is attenuated when attention is drawn away from the stimulus (*33*). These findings suggest that the redirecting of attention toward an annoying stimulus can be measured by the startle reflex.

Figure 5 depicts differences between startle electromyographic amplitudes determined during the four ETS episodes and their directly preceding zero-air condition. All EMG signals have been normalized by the startle amplitude of the first zero episode. Log-pPAH concentrations correlated nearly significantly to EMG amplitudes (negative *r*) when rank orders ($p = 0.058$) were not considered. Although we observed a negative trend as sETS concentrations increased, a significant difference existed only between

Table 3. Average concentrations of the continuously monitored environmental parameters during four exposure episodes depicted as sETS flow rates.

Environmental parameters	1st Zero	200 mL/min	2nd Zero	500 mL/min	3rd Zero	1,200 mL/min	4th Zero	3,600 mL/min
Particle numbers (cm^3)	0.02 (0.03)[a]	468 (110)	0.03 (0.03)	1,456 (266)	0.04 (0.04)	3,860 (795)	0.05 (0.04)	17,343 (1.891)
PPAH (ng/m^3)	1.6 (0.9)	9.3 (2.4)	1.8 (1.0)	22.8 (4.2)	2.1 (0.9)	58.5 (9.9)	2.2 (0.9)	218.8 (32.0)
CO (ppm)	0.16 (0.08)	0.21 (0.10)	0.16 (0.08)	0.27 (0.10)	0.15 (0.07)	0.39 (0.10)	0.15 (85)	1.07 (370)
tVOC (ppb)	1,244 (58)	1,256 (56)	1,238 (56)	1,282 (52)	1,232 (59)	1,340 (54)	1,231 (64)	1,702 (137)
CO_2 (ppm)	636 (79)	628 (44)	630 (62)	632 (83)	636 (93)	648 (61)	622 (74)	639 (46)
Temperature (°C)	23.8 (3.4)	24.0 (3.0)	23.8 (2.9)	23.8 (2.8)	23.9 (2.7)	23.9 (2.8)	23.9 (2.8)	23.7 (2.9)
Relative humidity (%)	27.9 (3.8)	27.2 (3.3)	27.4 (3.6)	27.3 (3.4)	27.1 (3.4)	27.3 (3.5)	27.0 (3.2)	27.1 (3.5)
ETS-$PM_{2.25}$[b] (µg/m^3)	0.7 (0.0)	5.1 (1.4)		34.0 (5.4)		115.5 (31.6)		430.7 (96.4)

The 1st, 2nd, 3rd, and 4th zero correspond to conditions without sETS exposure (compare to Figure 2).
[a]SDs in parentheses. [b]$PM_{2.25}$ measurements were performed on a separate occasion with the quartz crystal cascade impactor while the exposure chamber was vacant.

the highest concentrated sETS episode and its preceding zero condition (pairwise *t*-test, $p < 0.05$).

IAQ acceptability and ventilation requirements. Because detection of an sETS odor can be the key factor for indoor air quality acceptability, the question arises of how much fresh air is needed to dilute the sETS emissions of one cigarette to concentrations where no odor would be perceived. We divided sETS emissions per cigarette depicted in Table 2 by median odor threshold concentrations (Figure 3; while noses remained in the ducts). Thus, we obtained dilution volumes per sETS indicator. We then calculated the average dilution volumes based on volumes obtained from particle number, pPAH, and tVOC concentrations. To correct for coagulation and adsorption, we multiplied particle numbers by a factor of 5.7 (see "Cigarette Emission Experiment"). We assumed that the mass of the sETS emissions per cigarette is homogeneously distributed within a compartment and that no sinks are present. This produced an average fresh air volume of > 19,000 m^3 per cigarette in order to dilute to sETS concentrations where no odor would be perceived.

By the same method we observed eye and nasal irritations at dilution volumes corresponding to 3,000 m^3 per cigarette (lowest sETS concentration episode). At these sETS concentrations, 67% of the occupants judged the air unacceptable.

Breathing patterns and eye blink rates. Breathing pattern parameters (inhalation volume and inhalation flow rate) used as markers for olfactory or trigeminal activation (*34,35*) did not show any significant decrease during ETS exposure. There was a positive yet insignificant correlation between eye blink counts and log-transformed ETS particle concentrations.

Discussion

Cigarette Emission Experiment

Compared to other investigations, particle mass emissions observed in this study are about half as high as stated in the literature (*8,10*). This result is caused partly by the circumstance that our study measured not RSP (aerodynamic diameter of 3.5 µm) but $PM_{2.25}$. Furthermore, the cigarettes were not actively smoked but smoldered passively. The absence of exhaled mainstream smoke can reduce particulate matter of ETS by 15–43% (*36*). As for CO, concentrations are about 50% higher than reported by Martin and colleagues (*9*), whereas $tVOC_{FID}$ concentrations are approximately four times higher than reported by the same authors, possibly caused by the longer burning time of the cigarette that extinguished passively in our experiment. The greater relative contribution of tVOC measured in propane equivalents may result from organic compounds emitted from the smoldering filter material.

Olfactory Experiment

We hypothesize that the observed increase in sensitivity of the odor threshold while the subjects' noses remained in the olfactometer ducts compared to when the subjects' noses were placed into the ducts only upon presentation of the stimuli originates from an increase in mental concentration. Compared to an odor threshold variability of several orders of magnitude reported for some single chemicals (*37*), the variability of the observed sETS odor thresholds not exceeding a maximum value of 300 are low.

Odor thresholds of sETS obtained from the olfactory experiments showed that a median odor sensation was perceived at very low concentrations equivalent to an ETS-$PM_{2.25}$ concentration of approximately 0.6–1.4 µg/m^3. Because the olfactory stimuli were presented in ascending order, odor threshold values obtained in this experimental setting are considered to be the lowest attainable. The absolute values of these thresholds in terms of particle numbers, tVOC, and pPAH concentrations point out that, for field settings, an odor sensation would lie in the noise of the background concentrations. Typical long-term average concentrations reported in indoor settings where smoking takes place (*10,11*) are two orders of magnitude higher than concentrations at these threshold values. Compared to short-term concentrations, however, the determined odor threshold concentrations is up to three or more orders of magnitude lower than reported in field settings (*10,15,38*). The reason for the low threshold values found here is most likely the fact that our reference fresh air was cleared by an ultrafine particle filter and by an active carbon filter (see Figure 1).

Regarding the VOCs that can induce an odor sensation at concentrations near the determined odor threshold values, published odor thresholds for single chemicals suggest that not many compounds would be able to produce these thresholds (*39,42*). Among them, only pyridine could potentially create an odor sensation provided that minimum reported odor threshold values are taken as a criterion. This leads to the conclusion that other, perhaps unidentified compounds with an odor threshold in the nanogram or even picogram per cubic meter range could be responsible for the observed odor sensations. Furthermore, particles may be able to facilitate an odor sensation. Cain and colleagues (*8*) observed a slight decrease in odor intensity when ETS particles were electrostatically precipitated.

Full-Body Exposure Experiment

Environmental conditions. Based on the cigarette emission experiment, the highest episode concentration the subjects were exposed to is equivalent to one cigarette being smoked in a room about 100 m^3 in volume. Particle numbers concentrations averaged $1.7 \times 10^4/cm^3$; pPAH concentrations averaged 218 ng/m^3. Although these indicators are not typically assessed in ETS exposure studies, these values correspond to measurements obtained in field settings. A study performed by Morawska et al. (*39*) measured particle numbers of 5×10^4 at a rock concert. Junker et al. (*40*) reported pPAH concentrations of 336–990 ng/m^3in buildings for recreational activities. The lowest episode concentration is equivalent to one cigarette being smoked in a space of about 3,000 m^3, given a homogenous distribution of the emission. The average particle number and pPAH concentrations measured 468/cm^3 and 9.3 ng/m^3, respectively. As discussed above, the absence of exhaled mainstream ETS in this study underestimates the particulate exposure concentrations of the subjects compared to field settings (*36*). The gas-phase constituents of exhaled mainstream smoke, however, contributes only a small amount to ETS (*36*), so discrepancies in field settings are assumed to be small.

Table 4. Average perceived sensory responses of the sETS condition at a flow rate of 200 mL/min and the preceding zero condition.

Response	Symptom at zero air condition	Symptom at 200 mL/min
Temperature (–3, 3)	–0.56	–0.53
Relative humidity (–3, 3)	0.61	0.79
Odor strength (0, 6)	0.65	2.09#
Eye irritation (0, 6)	0.61	0.97*
Throat irritation (0, 6)	0.82	1.49**
Nasal irritation (0, 6)	0.55	0.94**
Arousal (0, 6)	0.41	1.79#
Annoyance (0, 6)	0.44	1.94#
Odor perception (–1, 1)	0.06	–0.22#
Air quality acceptability (–1, 1)	0.58	–0.03#
Percent acceptable	92	33#

The values in the parentheses correspond to the minimum and maximum values referred to on the vertical scale (Table 1). *$p < 0.05$, **$p < 0.01$, and #$p<0.001$, based on a pairwise *t*-test. Values of *p* for higher flow rates (not shown) are even lower.

Compared to field settings, the observed coagulation and particle removal processes overestimated the geometric mean diameters of the sETS aerosol. It has been reported that geometric mean diameters of ETS 10 min after having been generated by a human smoker increase 20–50% (*39*). In this study, the geometric mean diameter of the aerosol doubled, probably because the initial particle number concentration within the glove box is greater than would be measured in the field. Additionally, the interaction of small sETS particles with other surfaces would likely be larger than in a typical field setting.

Cain et al. (*8*) reported that the types of cigarettes generating sidestream smoke may create variations in the concentrations of ETS constituents. However, Nelson et al. (*43*) observed that ETS generated from a mix of the most widely used cigarette types is not significantly different from one country to another. The cigarette brands used in this study were chosen on the basis of sales statistics of the Swiss Community of the Cigarette Industry (*21*) and therefore represent ETS similar to that generated in other countries.

Sensory symptoms, startle reflex measurements, and eye blink rates. Because significant perceived sensory symptoms were observed at the lowest sETS exposure tested in this study, we conclude that thresholds of perceived sensory symptoms are even lower. Observed concentrations facilitating eye, nasal, and throat irritations correspond to an estimated ETS–$PM_{2.25}$ concentration of about 4.4 µg/m^3. This is equivalent to a dilution volume of about 3,000 m^3 per cigarette. Before this study, similar findings were reported at an ETS–RSP concentration of 58 µg/m^3 (*7*), although significant nasal irritations were not observed.

Only a few studies investigated the effect of odors on the startle reflex. Ehrlichman et al. (*44*) and Miltner et al. (*30*) investigated acoustic startle reflex modulation during short exposure to pleasant and unpleasant odors. Unpleasant odors enhanced startle amplitude, whereas pleasant odors had no effect. Later work (*45*) provided some evidence that a decreased startle reflex resulted from pleasant odors. These findings agree with the interpretation of Lang et al. (*29*) that the startle reflex amplitude is modulated by the emotional valence of the foreground stimulus. In contrast, we found a dose-dependent decrease in startle reflex amplitude with increasing concentrations of ETS. The differences between previous results and those of our study lie in the duration of the presented stimulus and in the analysis technique. Ehrlichman and Miltner presented the foreground odor stimulus for a very short period (one sniff) as Lang did with slides, rated high or low in valence. Startle amplitude was analyzed between the different trials only. We analyzed the difference in startle amplitude between, before, and during ETS stimulation, separately for each ETS concentration. Schicatano and Blumenthal (*33*) showed that distracting attention by attending to a visual search task reduced acoustic startle response amplitude. Therefore, we interpret our finding of a dose-dependent decrease of startle reflex amplitude as a directing of attention toward the increasing concentration of ETS.

Significant eye blink increases have been reported at concentrations > 1.3 ppm CO (*46*) and have been observed to increase in time (*5,7*). In this study, the concentration level as well as the duration of the episodes was not sufficient to create a significant increase in eye blink rates.

IAQ acceptability and ventilation requirements. Cain et al. (*8*) found that the degree of dissatisfaction evoked from ETS, strongly correlated to the perceived intensity of irritation or odor, depends on the channel (eye, nose, throat, odor) most severely affected. We found that the detection of the arousing sETS odor alone was sufficient to create dissatisfaction. However, dissatisfaction was not facilitated by the intensity of the perceived irritation, mainly because the sETS concentrations our subjects were

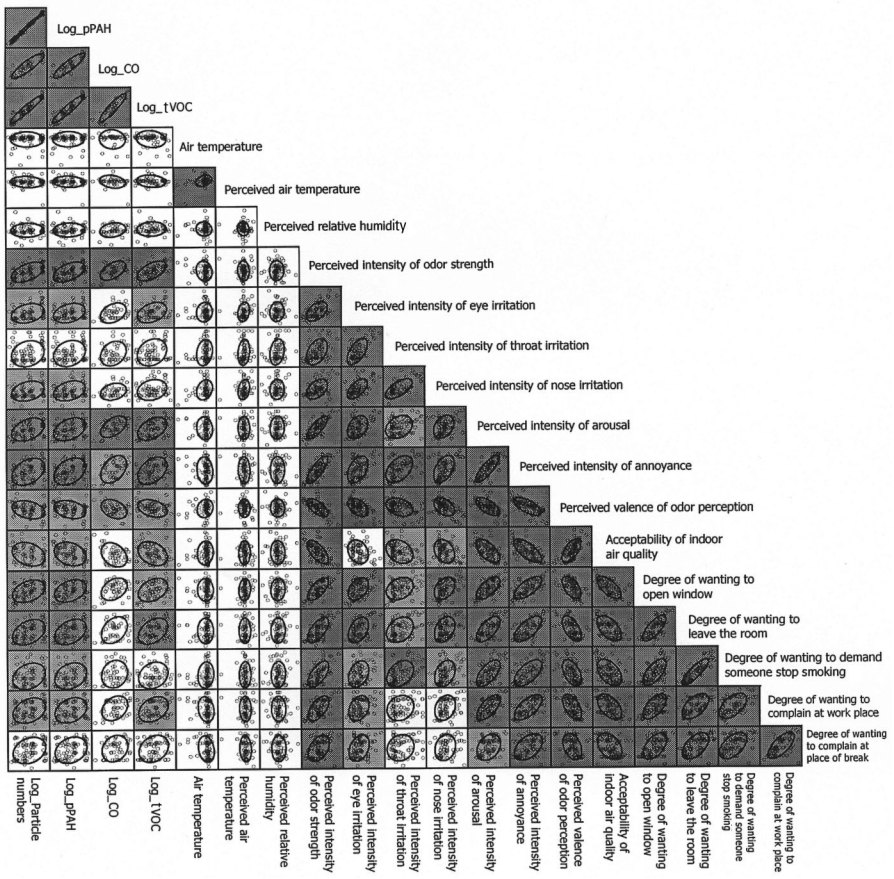

Figure 4. Scatter plots of background-corrected sensory responses (response at an ETS concentration episode minus response at the preceding zero concentration episode) and log-transformed ETS concentrations of 24 exposed subjects. The data depicted in the white boxes do not correlate significantly in a Pearson's linear regression model ($p > 0.01$). The data in the light gray boxes are highly significantly correlated to the linear trend ($p < 0.01$), and for the data in the dark gray boxes a very highly significant correlation exists ($p < 0.001$).

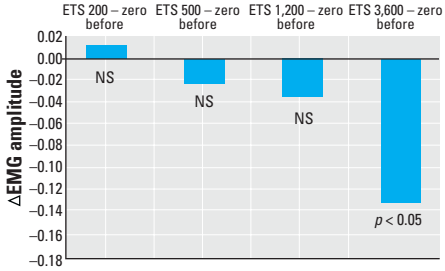

Figure 5. Differences between startle EMG amplitudes measured at an ETS condition (200 mL/min, 500 mL/min, 1,200 mL/min, and 3,600 mL/min) and the directly preceding zero-air condition for 22 subjects. The data of 22 of the 24 subjects were analyzed. Two data sets were rejected because they consisted of incomplete startle responses (this was possibly due to an inadequate placement of the electrodes onto the subject). The data have been normalized by EMG amplitudes measured during the first zero episode for each subject. NS, nonsignificant difference; significant difference determined by a pairwise *t*-test ($p < 0.05$).

exposed to were much lower than in the study of Cain and colleagues.

To create acceptable indoor air quality conditions, the sETS emissions of one cigarette would have to be diluted by an estimated fresh air volume of 19,000 m^3. This is at least two orders of magnitude higher than proposed by Cain et al. (*4*) for an estimated acceptability of 75–80% and an 80% acceptability by Walker and colleagues (*7*) in a full-body exposure study. These discrepancies are large. As stated above, the main reason is most likely the extremely clean reference air used in our study. Another factor may be that in our study a full-body exposure experiment was performed, whereas in the investigation of Cain et al. (*4*) subjects perceived the air at a sniffing station. Although these subjects did not smoke throughout the duration of the study, no information was given concerning their smoking status. Discrepancies with Walker et al.'s (*7*) study may emanate from the questions the subjects were asked about acceptability. Walker et al. employed a yes/no response to determine overall acceptance, whereas our study employed a voting scale ranging from clearly acceptable to just acceptable and from just unacceptable to clearly unacceptable. Studies by both Cain et al. (*4*) and Walker et al. (*7*) extrapolated the required fresh air volume (or the ETS concentrations) to where 80% of the subjects judged the quality of air to be acceptable. However, small changes in the slope of the log-scaled dose–response curves (ETS versus acceptability) will greatly influence the estimation of the of the 80% acceptability threshold. Obtained estimations must therefore be interpreted with great caution.

Controlled laboratory exposure studies conducted to date have not adequately considered low sETS concentrations that have adverse effects on perceived sensory symptoms. Furthermore, these studies have used ETS concentrations well above threshold concentrations of acceptable indoor air quality. To obtain realistic threshold concentrations for perceived sensory symptoms as well as acceptable indoor air quality, much lower exposure concentrations must be considered. In this study, we observed perceived sensory effects and a deterioration of indoor air quality at much lower sETS concentrations than previously reported. As Repace and Lowry (*48*) concluded, investigating cancer risk associated with ETS exposure, the degree to which ventilation rates would have to be increased to preserve indoor air quality in smoking areas would be impractical and economically unfeasible. We conclude that to protect nonsmokers effectively from adverse sensory symptoms and to provide acceptable indoor air quality, segregation of smoking and nonsmoking areas or smoking bans within public buildings should be enforced.

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Gaseous Pollutants in Particulate Matter Epidemiology: Confounders or Surrogates?

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Air pollution epidemiologic studies use ambient pollutant concentrations as surrogates of personal exposure. Strong correlations among numerous ambient pollutant concentrations, however, have made it difficult to determine the relative contribution of each pollutant to a given health outcome and have led to criticism that health effect estimates for particulate matter may be biased due to confounding. In the current study we used data collected from a multipollutant exposure study conducted in Baltimore, Maryland, during both the summer and winter to address the potential for confounding further. Twenty-four-hour personal exposures and corresponding ambient concentrations to fine particulate matter ($PM_{2.5}$), ozone, nitrogen dioxide, sulfur dioxide, and carbon monoxide were measured for 56 subjects. Results from correlation and regression analyses showed that personal $PM_{2.5}$ and gaseous air pollutant exposures were generally not correlated, as only 9 of the 178 individual-specific pairwise correlations were significant. Similarly, ambient concentrations were not associated with their corresponding personal exposures for any of the pollutants, except for $PM_{2.5}$, which had significant associations during both seasons ($p < 0.0001$). Ambient gaseous concentrations were, however, strongly associated with personal $PM_{2.5}$ exposures. The strongest associations were shown between ambient O_3 and personal $PM_{2.5}$ ($p < 0.0001$ during both seasons). These results indicate that ambient $PM_{2.5}$ concentrations are suitable surrogates for personal $PM_{2.5}$ exposures and that ambient gaseous concentrations are surrogates, as opposed to confounders, of $PM_{2.5}$. These findings suggest that the use of multiple pollutant models in epidemiologic studies of $PM_{2.5}$ may not be suitable and that health effects attributed to the ambient gases may actually be a result of exposures to $PM_{2.5}$. *Key words*: **air pollution, carbon monoxide, confounding, exposure error, personal exposure, $PM_{2.5}$, nitrogen dioxide, ozone, sulfur dioxide.** *Environ Health Perspect* **109:1053–1061 (2001). [Online _____]**
http://ehpnet1.niehs.nih.gov/docs/2001/109p1053-1061sarnat/abstract.html

Daily variations in air pollution have been associated with daily variations in deaths and hospital visits in a large number of locations around the world (*1–3*). Of the criteria air pollutants, the strongest and most consistent associations have been found for ambient particulate matter. Because ambient particle levels are often correlated with ambient concentrations of other gaseous pollutants, it is possible that the observed associations between particles and adverse health effects may be due to confounding by other correlated pollutants and not to the fine particles themselves (*4,5*).

The issue of confounding in air pollution epidemiology has been examined in several large multicity studies (*6,7*). These studies proceeded on the assumption that the best way to assess the independent effects of two or more pollutants is to include the pollutants in the regression model at the same time. Samet et al. (*6*), for example, analyzed ambient air pollution [particulate matter ≤ 10 μm (PM_{10}), ozone, nitrogen dioxide, carbon dioxide, and sulfur dioxide] and daily mortality data from 20 cities with varying pollution profiles and found PM_{10} to be a significant predictor of daily mortality controlling for the gaseous copollutants. Schwartz (*7*) examined 10 cities separately during the summer and winter and reported identical associations between daily mortality and PM_{10}. Because the relationship among ambient PM_{10} and its copollutants differed substantially by season, the observed identical summer and winter associations were offered as compelling evidence that particle associations were not affected by confounding from other pollutants. Similarly, Fairley (*8*) examined the relationship between ambient $PM_{2.5}$, PM_{10}, $PM_{2.5–10}$, sulfate, CO, O_3, and NO_2 and corresponding mortality. Fairley observed significant associations for numerous pollutants when the pollutants were examined individually. When the gaseous pollutants were examined along with $PM_{2.5}$, the significant associations for the gases disappeared, while the association for $PM_{2.5}$ became stronger; this suggests that fluctuations in ambient $PM_{2.5}$ concentrations are driving the health effect associations. All of these epidemiologic studies conducted to date, however, have investigated the potential for confounding using ambient pollutant concentrations, as none were able to include information about the personal exposures to the various air pollutants.

Information concerning personal exposures is critical to our ability to determine whether confounding is a potential problem within epidemiologic studies. The coefficient for $PM_{2.5}$ represents the independent effect of particles controlling for the other pollutant in a two-pollutant model, if each ambient pollutant measurement is a surrogate for actual exposures to that same pollutant. We began to examine the relationship between ambient pollutant concentrations and corresponding personal exposures and its copollutants in our exposure study of older adults living in Baltimore, Maryland (*9*). Results from this study showed that, despite significant associations among the ambient pollutant concentrations, personal exposures to $PM_{2.5}$ were not significantly correlated with personal exposures to any of its copollutants, including O_3, NO_2, and $PM_{2.5–10}$. Moreover, personal $PM_{2.5}$ exposures were significantly associated with its corresponding ambient concentrations, but the personal ambient associations were not significant for O_3, NO_2, or $PM_{2.5–10}$. These findings suggest that for this Baltimore cohort, true confounding of $PM_{2.5}$ by its copollutants is implausible and that ambient $PM_{2.5}$ concentrations are reasonable surrogates of their personal $PM_{2.5}$ exposures.

In this study, we further evaluated the role of ambient O_3, NO_2, SO_2, and CO as confounders of ambient $PM_{2.5}$ using data from the Baltimore study of older adults and using additional data collected in Baltimore for individuals with chronic obstructive pulmonary disease (COPD) and children. Our goal, in particular, was to understand for which exposure each ambient measurement was a surrogate.

Methods

Personal multipollutant exposures and corresponding ambient concentrations were measured for 56 subjects (three cohorts: 20 older adults, 21 children, and 15 individuals with COPD) living in the metropolitan Baltimore

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We thank the participants of this study as well as J. Evans and P. Koutrakis for their valuable insight and feedback. Ambient data were provided, in part, by the Maryland Department of the Environment.

This study was supported by the Health Effects Institute (award 98-7), Harvard-EPA Center on Particle Health Effects (grant R827353-01-0), the Electric Power Research Institute, and the American Petroleum Institute.

Received 30 January 2001; accepted 5 April 2001.

area. All subjects included in this analysis were nonsmokers and lived in nonsmoking private residences (i.e., either single-family houses or apartments). Sampling was conducted during the summer (29 June–23 August 1998) and winter (2 February–13 March 1999). Fourteen of 56 subjects participated in both sampling seasons. During both the summer and winter sampling periods, subjects included older adults and children. Subjects from the older adult cohort consisted of retired, healthy adults with an average age (± SD) of 75 ± 6.8 years. Subjects from the children's cohort consisted of healthy schoolchildren between 9 and 13 years of age. During the winter, personal exposures for individuals with COPD were also measured along with the older adults and children. Subjects from the COPD cohort consisted of individuals with physician-diagnosed moderate-to-severe COPD with an average age of 65 ± 6.6 years. Although the subjects were from a range of socioeconomic backgrounds and geographic locations within Baltimore, subject selection was random and was not intended to be representative of sensitive populations in general. Subjects completed and returned informed consent forms before their participation in the study.

All subjects were monitored for 12 consecutive days in each of the one or two seasons, with the exception of children who, during the summer, were measured for 8 consecutive days. We measured 4–16 subjects during each 12-day monitoring period. A total of 800 person-days of exposure data were collected for some of the following pollutants: $PM_{2.5}$, PM_{10}, O_3, NO_2, SO_2, elemental carbon (EC), organic carbon (OC), and volatile organic compounds (VOCs; Table 1). Because PM_{10} and VOCs were only sampled for the older adult cohort and there were questions concerning the precision of the OC measurements, these exposures were not included in this analysis.

A subset of $PM_{2.5}$ filters was analyzed for SO_4^{2-} concentration. For these filters, personal exposure to $PM_{2.5}$ of ambient origin was estimated using the expression:

$$\left(\frac{\left[SO_4^{2-}\right]_{\text{personal}_{ij}}}{\left[SO_4^{2-}\right]_{\text{ambient}_j}}\right) \bullet \left[PM_{2.5}\right]_{\text{ambient}_j}$$

where personal_{ij} represents the personal exposure to SO_4^{2-} for subject i on day j, and ambient_j represents the ambient concentration measured at the stationary site on day j. The effective penetration of ambient $PM_{2.5}$ to personal exposures for all fine particles was assumed to equal that for SO_4^{2-}. Since recent studies have shown that fine particle deposition rates and penetration efficiencies vary by particle size and other factors such as air exchange rates (*10*), SO_4^{2-}-based estimates used in the current study provide only an indication of exposure to $PM_{2.5}$ of ambient origin rather than a definitive value. With the exception of NO_2, the gaseous copollutants measured during the study were primarily (if not exclusively) ambient in origin. To estimate exposures to NO_2 of ambient origin, analyses involving personal NO_2 exposures were performed by controlling for the potential nonambient contributions from gas stoves, the primary nonambient source of NO_2 for these cohorts.

Personal exposure samples were collected using a specially designed multipollutant sampler that consisted of personal environmental monitors (PEMs) to collect $PM_{2.5}$, PM_{10}, EC, and OC; sorbent tubes filled with activated carbon to collect VOCs; and passive samplers to collect O_3, NO_2, and SO_2. Subjects were permitted to remove the sampler during prolonged periods of inactivity (i.e., sleeping, watching television) and during activities when the sampler could be damaged (i.e., showering, intense physical activity). When the sampler was removed from the subject's body, subjects were instructed to keep the sampling inlets as close as possible to their breathing zone. The design and performance of this sampler have been described, in detail, elsewhere (*9,11*).

We measured 24-hr integrated ambient $PM_{2.5}$ and PM_{10} concentrations using Harvard Impactors at a centrally located site. Continuous ambient $PM_{2.5}$ mass concentrations were obtained from a pair of $PM_{2.5}$ tapered element oscillating microbalances (TEOMs; model 1400A; Rupprecht & Patashnick, Co., Inc., Albany NY) operated by the Maryland Department of the Environment. Ambient O_3, NO_2, SO_2, CO, and VOC data were obtained from local stationary ambient monitoring sites operated by the Maryland Department of the Environment for monitoring citywide pollutant concentrations. Additional ambient $PM_{2.5}$ concentrations were obtained from the U.S. Environmental Protection Agency that was collected as part of a personal exposure study (*12*). O_3, NO_2, SO_2, and CO were measured using UV photometric analyzers, chemiluminiscence monitors, pulsed fluorescent monitors, and nondispersive infrared monitors, respectively. All of the participants' residences were located within an approximately 40-km radius from each of the stationary sites which were located either within the city of Baltimore or Baltimore County. $PM_{2.5}$ concentrations were obtained from the Old Town monitoring station; O_3 from the Living Classroom, and Essex monitoring stations during the summer and from the Essex monitoring stations during the winter; NO_2 from the Old Town, Living Classroom, and Essex stations during the summer and from the Old Town and Essex stations during the winter; SO_2 from the Rivera Beach monitoring station; and CO from the Old Town monitoring station. In cases where pollutant concentrations were measured at multiple sites, concentrations were averaged across the sites. Additional data collected included daily time–activity diaries and household characteristic surveys that provided supplemental information relating to pollutant exposures.

Standard quality assurance procedures were followed for this study (*13*). We assessed collected data for bias, precision, and completeness. Completeness for personal $PM_{2.5}$, O_3, NO_2, SO_2, SO_4^{2-}, and EC was 92, 83, 90, 91, 91 and 91%, respectively. Completeness for the ambient pollutant concentrations was > 98% for all of the sampled pollutants. Precision, accuracy, and limit of detection information are detailed in Chang et al. (*11*) and Sarnat et al. (*9*). All samples were field-blank corrected. Teflon PEM filters were also corrected for barometric pressure.

Sampler measurement error (sampler error) was calculated by collocating replicate, fully configured sampling packs for 24 hr (± 10%). Sampler error was estimated as the root mean squared difference of the collocated samplers, divided by the square root of two, divided by the mean concentration of the samples. Based on precision data from this study and previous studies, we assumed that precision was relative and that sampler error values for the outdoor range of concentrations applied to the entire range of personal exposure concentrations (*9*).

Correlation of sampler error in the dependent and independent variables was assumed to be independent of each other, a valid assumption based on previous laboratory and field characterization tests (*14*). In univariate regression analysis (such as the mixed-model approach used in the current analysis) sampler error in the dependent

Table 1. Sampling plan.

Cohort	Older adults	COPD	Children
Season			
Summer (*n*)	15		10
Winter (*n*)	15	15	15
Sampling duration (days and season)	12	12	8 (summer) 12 (winter)
Pollutants			
$PM_{2.5}$	√	√	√
PM_{10}	√		
O_3	√	√	√
NO_2	√	√	√
SO_2	√	√	√
VOCs	√		
EC/OC		√	√(winter only)
CO	(ambient measurements only)		

variable may lead to biased correlations between the variables but will not bias the estimates of slope or intercept (*15*). Sampler error in the independent variable, on the other hand, may bias estimates of the slope and intercepts as well as reduce model sensitivity. To account for the effects of this error, we corrected the slope by adjusting the variance associated with the sampler error:

$$\hat{\beta}_{\text{true}} = \hat{\beta}_{\text{obs}}\left(\frac{\sigma^2_{\text{obs}}}{\sigma^2_{\text{true}}}\right), \quad [1]$$

where $\hat{\beta}_{true}$ is the slope of the regression corrected for sampler error, $\hat{\beta}_{obs}$ is the slope of the observed or naïve regression results, $\hat{\sigma}^2_{\text{obs}}$ is the variance of the observed exposures or concentrations, and $\hat{\sigma}^2_{\text{true}}$ is the estimated observed variance of the exposures or concentrations minus the estimated variance attributable to sampler error. The true standard error of the mixed-model slope (i.e., the estimated standard error minus the fraction attributable to sampler error) can be estimated using the delta method, which is expressed in Equation 2 (*15*) where $\hat{SE}(\hat{\beta}_{\text{true}})$ is the estimated standard error of the true slope of the regression, $\hat{Var}(\hat{\beta}_{\text{true}})$ is the estimated variance of the true slope of the regression, and $\hat{Var}(\hat{\beta}_{\text{obs}})$ is the estimated variance of the observed slope of the regression. The true significance of the slope was subsequently determined as the $(\hat{\beta}_{\text{true}})$ divided by $\hat{SE}(\hat{\beta}_{\text{true}})$.

Data analysis. Units for $PM_{2.5}$, SO_4^{2-} and EC concentrations and exposures are reported in micrograms per cubic meter. Units for O_3, NO_2, and SO_2 concentrations and exposures are reported in parts per billion. Units for CO concentrations and exposures are reported in parts per million. Negative values for the gaseous pollutants as well as values less than their respective limits of detection were included in the data analyses as measured to avoid bias in estimating relations among measurements (*16*). Graphical techniques and Shapiro-Wilks tests for normality indicated that most of the pollutants were normally or nearly normally distributed.

We examined four sets of associations to assess the relationship between $PM_{2.5}$ and its copollutants, including the association between *a*) ambient $PM_{2.5}$ concentrations and ambient copollutant concentrations; *b*) ambient pollutant (both $PM_{2.5}$ and copollutants) concentrations and their respective personal exposures; *c*) personal $PM_{2.5}$ exposures and personal copollutant exposures; and *d*) ambient copollutant concentrations and personal $PM_{2.5}$ exposures. In addition, models using $PM_{2.5}$ components, such as SO_4^{2-}, EC, and $PM_{2.5}$ of ambient origin were examined to identify factors that may affect the above associations.

Analyses of the associations between ambient $PM_{2.5}$ concentrations and ambient pollutant concentrations were conducted using univariate time-series regression analysis assuming a first-order autoregressive structure for the error. Because personal exposures were measured repeatedly for each subject, analyses of personal exposure data were conducted using mixed models and individual-specific Spearman's correlation coefficients (r_s). Pollutant exposures and concentrations were modeled as fixed-effects variables, and subjects were modeled as random variables to account for between subject variation. Models were fitted using a compound symmetry covariance matrix which yielded the lowest Akaike Information Criteria diagnostic values compared with other covariance matrices examined (e.g., autoregressive, banded toeplitz). Data from the three cohorts were analyzed in aggregate, with the exception of cases where significant differences in associations among the cohorts were found. It should be noted that, due to the intrasubject correlation, coefficients of determination (R^2) or other measures of scatter are not statistically valid and are, therefore, not reported. Consequently, strength of association was determined by the significance of the slope of the mixed models. Distributions of individual-specific r_s values are also reported as another indicator of the strength of the observed associations. The primary objective of the analysis was to examine the predictive power of a single pollutant exposure or concentrations for other exposures or concentrations. Therefore, the models are almost exclusively univariate models with the sole exception being models that control for the impact of indoor NO_2 contributions from gas stoves, which have a cooking-fuel interaction term. All of the above analyses were computed using SAS software (SAS Institute, Cary, NC). Statistical significance is reported at the 0.05 level unless otherwise specified.

$$\hat{SE}\left(\hat{\beta}_{\text{true}}\right) = \sqrt{\hat{Var}\left(\hat{\beta}_{\text{true}}\right)} = \sqrt{\left(\frac{\hat{\sigma}^2_{\text{obs}}}{\hat{\sigma}^2_{\text{true}}}\right)^2 \hat{Var}\left(\hat{\beta}_{\text{obs}}\right) + \left(\frac{\hat{\beta}_{\text{obs}}}{\hat{\sigma}^2_{\text{true}}}\right)^2 \hat{Var}\left(\hat{\sigma}^2_{\text{obs}}\right) + \frac{\left(\beta_{\text{obs}} \cdot \hat{\sigma}^2_{\text{obs}}\right)^2}{\left(\hat{\sigma}^2_{\text{true}}\right)^4} \hat{Var}\left(\hat{\sigma}^2_{\text{true}}\right)}$$

Equation 2.

Exclusion of data points. Data points were voided due to sampling problems (e.g., pump or battery failures, tube disconnection) or laboratory analysis irregularities. Time–activity data indicated that two subjects (one older adult who participated during both sampling periods and one child who participated during the summer sampling period) were heavily exposed to environmental tobacco smoke (ETS) throughout the course of their participation in the study. Days of heavy or prolonged exposure to ETS were not included in the analyses, since collected samples did not typify exposures for a nonsmoker or someone living in a residence with nonsmokers.

Results

Summary statistics for the measured ambient concentrations and personal exposures, stratified by season and by cohort are presented in Figure 1. A summary of household characteristic and time activity data is presented in Table 2. In general, cohort-specific differences in household characteristics and time–activity patterns were not apparent, which may be due to the relatively small size of each cohort. There were, however, a number of observed differences that varied by cohort, but these were probably not specifically related to cohort affiliation. Most of the monitored children and individuals with COPD lived in single-family houses (35 of 40 subjects), whereas subjects from the older adult cohort lived equally in apartments (18 of 30 subjects) and single-family homes. Approximately one-half of the subjects (34 of 69) lived in residences with gas stoves, a potential source of NO_2 and CO, although few participants spent substantial periods of time cooking. Time–activity diary results showed that older adult subjects spent less than 2% of the day, on average, engaged in stove-related cooking activities. Only three of the subjects lived in residences with attached garages, another potential source of $PM_{2.5}$, CO, and NO_2. Similarly, there were approximately an equal number of subjects from each cohort living near (100 yards) busy roads. Few subjects indicated on their time–activity diaries any exposure to ETS during their respective sampling periods. Older adults and children spent similar fractions of time outdoors during the summer (4.7% and 5.7% of the day, respectively). Time spent outdoors during the winter was not examined but was assumed to be limited for all subjects.

Are ambient copollutant concentrations significantly associated with ambient $PM_{2.5}$ concentrations? Significant associations were found between ambient $PM_{2.5}$ and corresponding ambient copollutant concentrations

during both the summer and winter. For O_3 and CO, the strength and the direction of this association varied by season (Tables 3 and 4). During the summer, ambient $PM_{2.5}$ was significantly and positively associated with ambient O_3 and NO_2 (r_s = 0.67 and 0.37, respectively). During the winter, ambient $PM_{2.5}$ was significantly and positively associated with ambient NO_2 and CO (r_s = 0.75 and 0.69, respectively). A significant, negative association was found between ambient $PM_{2.5}$ and O_3 during the winter (r_s = –0.72). Ambient $PM_{2.5}$ and SO_2 were not significantly associated during the winter (r_s = –0.17).

Are personal exposures to copollutants significantly associated with personal exposures to $PM_{2.5}$? In contrast to the ambient concentrations, virtually none of the personal copollutant exposures were significantly associated with corresponding personal $PM_{2.5}$ exposures (Table 5). The summertime association between personal $PM_{2.5}$ and NO_2 (slope = 0.18, $p < 0.01$) was the sole exception to this finding. There was some evidence that the strength of the personal $PM_{2.5}$–NO_2 association was largely driven by older adult subjects (slope = 0.21, p = 0.01), as results using data only from the children were not significant (slope = 0.06, p = 0.62). Conversely, although insignificant when data from all the cohorts were analyzed together, summertime personal $PM_{2.5}$ and O_3 were significantly associated for children (slope = 0.37, p = 0.03), but not for older adults (slope = 0.07, p = 0.73). The fraction of time spent outdoors during the summer differed little by cohort, so reasons for these cohort differences are not known but may result from different activity patterns.

Similar, yet slightly stronger, associations were found when personal exposures to $PM_{2.5}$ of ambient origin, as opposed to total $PM_{2.5}$, were regressed on personal copollutant levels (Table 5). During both the summer and winter, the significance of the slope (as evidenced by the *t*-statistics for the mixed model slopes) between personal $PM_{2.5}$ of ambient origin and both personal O_3 and NO_2 increased, as compared to models using total personal $PM_{2.5}$, but remained insignificant. Results from models that included a cooking-fuel interaction term showed that gas stoves did not significantly affect the strength of the personal $PM_{2.5}$–NO_2 associations (summertime p = 0.61; wintertime p = 0.44). During the summer, cooking fuel was shown to interact significantly with the strength of the association between personal exposure to $PM_{2.5}$ of ambient origin and personal NO_2 (0.02), with subjects living in residences with gas stoves having stronger associations as compared to those living in residences with electric stoves. Cooking fuel was not shown to influence the wintertime association between personal exposures to $PM_{2.5}$ of ambient origin and NO_2 significantly (p = 0.22).

An analysis of the individual-specific pairwise correlation coefficients showed similar weak associations between personal $PM_{2.5}$ and corresponding personal copollutant exposures. Only 9 of the 178 individual-specific pairwise correlations were significant (3 during the summer and 4 in the winter for $PM_{2.5}$–NO_2; 1 during the summer for $PM_{2.5}$–O_3; and 1 during the winter for $PM_{2.5}$–SO_2; Figure 2). Of these significant correlations, three between personal $PM_{2.5}$ and personal NO_2 were negative, an inverse relationship from that observed between the ambient concentrations of these two pollutants. Similar results were found for personal $PM_{2.5}$ of ambient origin. Of 115 total correlations examined using personal $PM_{2.5}$ of ambient origin, only 5 were significant.

Are ambient pollutant concentrations associated with their respective personal exposures? The weaker associations among the personal pollutant exposures as compared to associations among the ambient pollutant

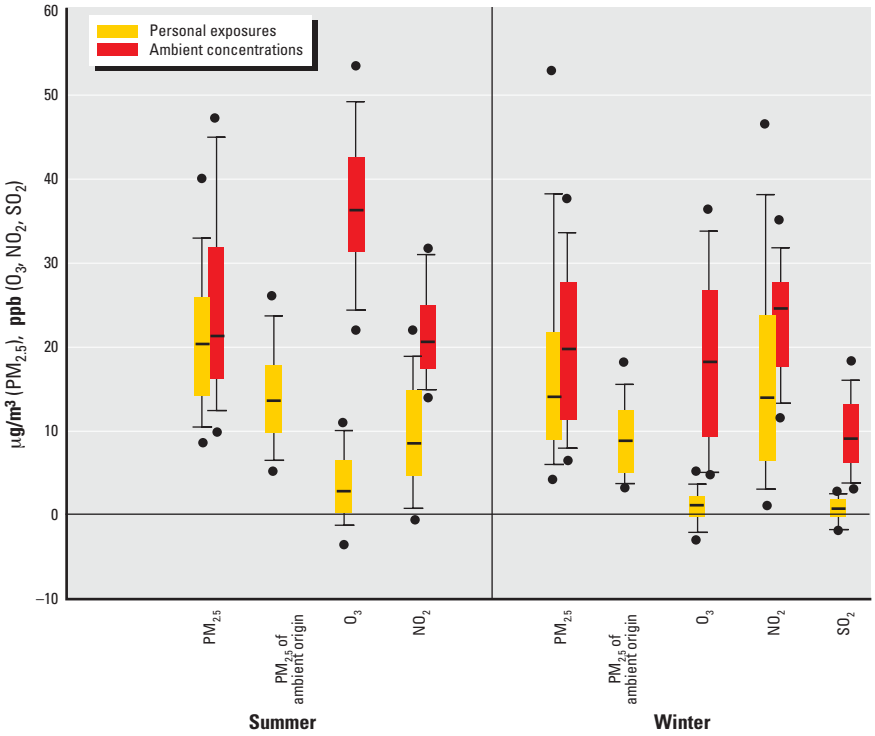

Figure 1. Boxplots showing the distribution (5th, 10th, 25th, median, 75th, 90th, and 95th percentiles) of ambient concentrations and personal exposures by season and pollutant.

Table 2. Summary of cohort-specific household characteristics and time–activity data.

	Older adults		Children		COPD
	Summer (n = 15)	Winter (n = 15)	Summer (n = 10)	Winter (n = 15)	Winter (n = 15)
Single-family houses	5	7	10	14	11
Gas stoves	4	8	5	8[a]	9
Attached garages	0	1	0	1[a]	1
Percentage of time outdoors	4.7%[b]	—	5.7%	—	—
Storm windows	—	10	—	8[a]	8
Near (100 yards) busy road	4	5	5	8[a]	7

[a]Includes data for 11/15 subjects only. [b]Includes data for 9/10 subjects only.

Table 3. Correlations among ambient concentrations (Spearman's *r*).

	$PM_{2.5}$	O_3	NO_2	SO_2	CO
$PM_{2.5}$	1.00	0.67*	0.37*	—	0.15
O_3	–0.72*	1.00	0.02	—	–0.06
NO_2	0.75*	–0.71*	1.00	—	0.75*
SO_2	–0.17	0.41*	–0.17*	1.00	–0.32*
CO	0.69*	–0.67*	0.76*	–0.12	1.00

Top right represents summertime correlations. Lower left represents wintertime correlations.
*Significant at the 0.05 level.

concentrations were not unexpected given that ambient concentrations for gaseous pollutants were not associated with their respective personal exposures (Table 6), as also shown in our previous paper (*9*) as well as in other exposure studies (*17,18*). Of the measured pollutants, $PM_{2.5}$ was the only pollutant for which ambient concentrations were significantly (and positively) associated with their respective personal exposures. (Although personal SO_2 was significantly associated in the winter with corresponding ambient concentrations, their association was negative: slope = –0.05, p = 0.005). The strong personal-ambient associations for $PM_{2.5}$ were found during both the summer and winter (p < 0.0001), providing further evidence of the strong longitudinal association between ambient $PM_{2.5}$ and corresponding personal exposures (*9,19,20*). Personal-ambient associations for personal $PM_{2.5}$ of ambient origin were similarly strong and with increased significance during the winter (the t-value rose from 3.56 to 14.11; Table 6). The presence of gas stoves did not significantly affect the personal-ambient NO_2 associations (summertime interaction with cooking-fuel type, p = 0.56; wintertime p = 0.57).

The interpersonal variability of the personal-ambient association varied by pollutant (Figure 2). For both seasons, the median correlation between ambient concentrations and personal exposures was highest for $PM_{2.5}$ (summer median r_s = 0.65, 13 of 24 significant correlations; winter median r_s = 0.22, 10 of 44 significant correlations). Even higher correlations were shown for SO_4^{2-}, a component of $PM_{2.5}$ that is predominantly of ambient origin (summer median r_s = 0.88, 13 of 14 significant correlations; winter median r_s = 0.71, 16 of 29 significant correlations). Among the gaseous copollutants, the wintertime personal-ambient association for NO_2 was the strongest with 7 of 44 subjects having significant correlations between ambient NO_2 and their personal NO_2 exposures.

Are ambient copollutants surrogates for personal exposure to $PM_{2.5}$? Although ambient copollutant concentrations were generally not associated with their respective personal exposures, they were associated with personal $PM_{2.5}$ during both seasons (Table 7). The sole exception was summertime ambient CO, which was not significantly associated with personal $PM_{2.5}$. The direction of the associations between personal $PM_{2.5}$ and the ambient copollutant concentrations mirrored those of the corresponding ambient associations between $PM_{2.5}$ and its respective copollutants. Results from cohort-specific models examining these associations were not consistently significant, which may be due to the relatively small sample size since the slope and intercepts were relatively stable. The children's summertime association between ambient O_3 and total personal $PM_{2.5}$ was the sole exception, being both insignificant (p = 0.99) and significantly different from results involving the older adults (p = 0.03).

The associations between ambient copollutant concentrations and personal $PM_{2.5}$ of ambient origin were consistently stronger than those for total personal $PM_{2.5}$. Additionally, all of the cohort-stratified associations between ambient copollutant concentrations and personal $PM_{2.5}$ of ambient origin were significant. [The wintertime association between ambient SO_2 and personal $PM_{2.5}$ of ambient origin for the older adults was significant, but at the 0.1 level (p = 0.09).] Furthermore, when associations were examined using maximum 1-hr averages for O_3 and CO instead of the integrated 24-hr averages of these pollutants, model results were comparable (Table 8). Finally, ambient $PM_{2.5}$ was not associated with exposures to any of its gaseous copollutants during either season.

Are ambient copollutant concentrations surrogates for personal exposure to $PM_{2.5}$ from specific sources? Personal EC and SO_4^{2-} were also measured during the winter for the cohort of COPD patients, and we used data from this cohort and season to identify factors that affected the association between the ambient copollutant concentrations and personal $PM_{2.5}$ exposures from different ambient sources (Table 9). Specifically, SO_4^{2-}, a secondary pollutant formed from coal-fired power plants, was used as a marker of regional pollution, and EC was used as an indicator of mobile source pollution. For the COPD cohort, ambient NO_2, SO_2, and CO were significantly associated with personal $PM_{2.5}$ of ambient origin with t-values that were consistently higher than those observed for models using exposure to total $PM_{2.5}$. These results suggest that personal exposures to the copollutants for this cohort were primarily surrogates for ambient particles. The associations between the ambient copollutants and the personal SO_4^{2-} and EC varied by pollutant. Personal SO_4^{2-} was significantly and negatively associated with ambient O_3 and SO_2 (p = 0.0009 and 0.0125, respectively), and personal EC was significantly associated with ambient O_3, NO_2, and CO (p < 0.0001 for all). This suggests that ambient O_3 is primarily a surrogate for secondary particle exposures, whereas ambient CO and NO_2 is primarily a surrogate for particles from traffic.

Estimating the effects of sampler measurement error on the results. The relative precision for a given sampler (i.e., the percentage of variability attributable to sampler and analytical error) varied by pollutant, season, and filter batch (Table 10). During the summer, relative precision for the personal exposure samplers was similar (range: 8% for $PM_{2.5}$ to 14% for NO_2), whereas during the winter the precision was more variable (range: 5% for $PM_{2.5}$ to 39% for NO_2). The relative precision of the ambient monitors (under 5% for all pollutants) was consistently lower than that observed for the personal samplers. Table 10 shows that although sampler error may have elevated the degree of overall variability in the exposures, true

Table 4. Association between ambient $PM_{2.5}$ concentrations and ambient copollutant concentrations.

Season	Model	No.	Slope	t-Value	Intercept
Summer	Ambient $PM_{2.5}$ = ambient O_3	48	0.84*	5.98	–5.61
Winter		37	–0.67*	–5.56	32.31*
Summer	Ambient $PM_{2.5}$ = ambient NO_2	48	0.65*	2.21	11.12
Winter		37	1.02*	6.22	–2.74
Summer	Ambient $PM_{2.5}$ = ambient CO	48	6.50	0.57	21.95*
Winter		37	15.93*	5.56	5.84*
Winter	Ambient $PM_{2.5}$ = ambient SO_2	37	–0.34	–0.93	23.05*

Estimates generated using time series regression analysis.
*Significant at the 0.05 level.

Table 5. Association between personal $PM_{2.5}$ exposures and personal copollutant exposures.

		Total personal $PM_{2.5}$ exposure				Personal exposure to $PM_{2.5}$ of ambient origin			
Season	Model	Subjects (n)	Slope	t-Value	Intercept	Subjects (n)	Slope	t-Value	Intercept
Summer	Personal $PM_{2.5}$ = personal O_3	24 (193)	0.21	1.31	19.78*	15 (130)	0.22	1.56	13.12*
Winter		45 (434)	–0.05	–0.20	18.51*	30 (282)	–0.18	–1.66	9.01*
Summer	Personal $PM_{2.5}$ = personal NO_2	24 (213)	0.18*	2.51	18.65*	15 (150)	0.17*	3.03	12.77*
Winter		45 (467)	–0.02	–0.68	19.04*	30 (289)	–0.16	–0.83	9.23*
Winter	Personal $PM_{2.5}$ = personal SO_2	45 (465)	–0.19	–0.65	18.68*	30 (289)	0.03	0.18	8.98*

*Significant at the 0.05 level.

variability in the exposures accounted for the majority of overall variability (> 66%), even for exposures whose mean concentrations were extremely low (e.g., O_3 and SO_2). These results suggest that true variability contributed more to the overall variability in exposures than sampler error. As a result, there was likely sufficient variability in exposures to detect significant associations when they truly existed.

Because sampler error increases the likelihood of type II errors, we conducted further analyses to quantify its effect on models with insignificant results. For models examining the association between ambient copollutant concentrations and personal $PM_{2.5}$ exposures, reduced model sensitivity was not likely to affect the interpretation of the results, as the slopes were highly significant in spite of any sampler error. Furthermore, the estimates of slope for the models examining

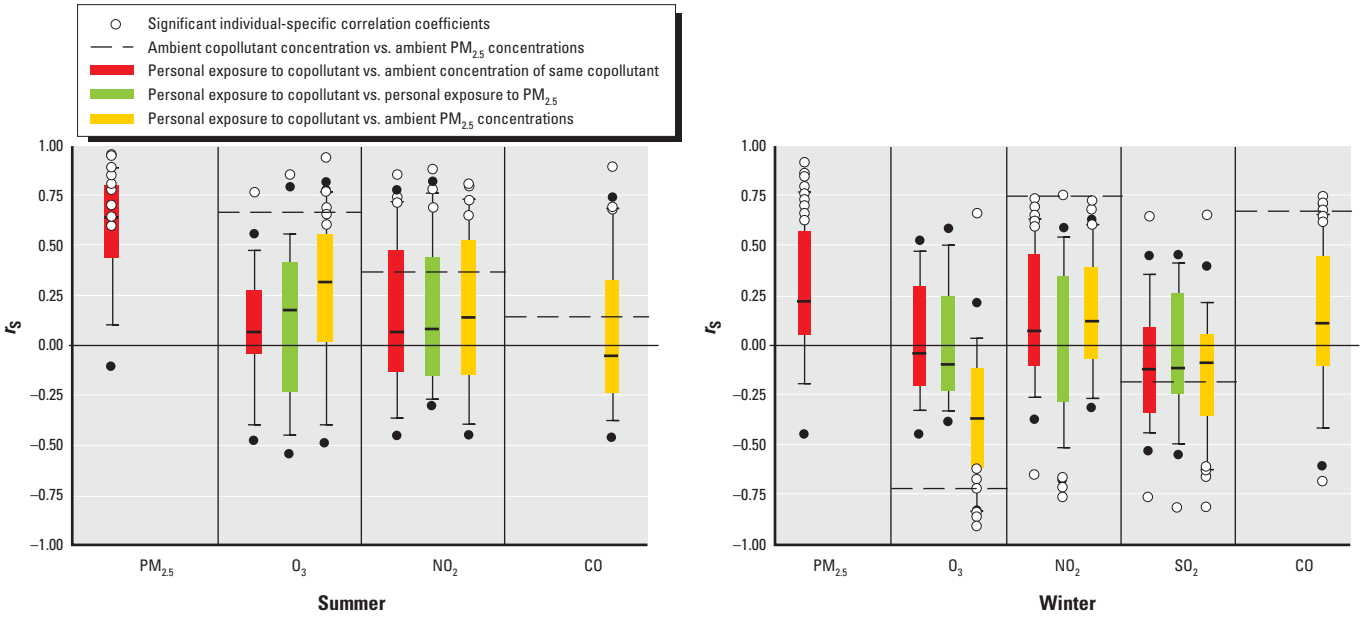

Figure 2. Boxplots showing the distribution (5th, 10th, 25th, median, 75th, 90th, and 95th percentiles) of individual specific Spearman's correlation coefficients (r_s) for (*A*) summertime and (*B*) wintertime plots (n = 24 and 44, respectively).

Table 6. Association between ambient concentrations and respective personal exposures.

		Total personal $PM_{2.5}$ exposure				Personal exposure to $PM_{2.5}$ of ambient origin			
Season	Model	Subjects (n)	Slope	t-Value	Intercept	Subjects (n)	Slope	t-Value	Intercept
Summer	Personal $PM_{2.5}$ = ambient $PM_{2.5}$	24 (225)	0.46*	9.96	10.20*	15 (154)	0.34*	11.12	5.56*
Winter		45 (481)	0.26*	4.36	13.27*	30 (301)	0.39*	19.88	1.19*
Summer	Personal O_3 = ambient O_3	24 (196)	0.01	1.21	1.84				
Winter		45 (449)	0.00	0.03	0.46				
Summer	Personal NO_2 = ambient NO_2	24 (217)	0.04	0.37	9.52*				
Winter		45 (484)	–0.05	–0.53	18.16*				
Winter	Personal SO_2 = ambient SO_2	45 (487)	–0.05*	–2.82	0.54*				

*Significant at the 0.05 level.

Table 7. Association between personal $PM_{2.5}$ exposures and ambient copollutant concentrations.

		Total personal $PM_{2.5}$ exposure				Personal exposure to $PM_{2.5}$ of ambient origin			
Season	Model	Subjects (n)	Slope	t-Value	Intercept	Subjects (n)	Slope	t-Value	Intercept
Summer	Personal $PM_{2.5}$ = ambient O_3	24 (225)	0.28*	4.00	10.94*	15 (150)	0.37*	6.23	0.04
Winter		45 (487)	–0.29*	–4.68	23.86*	30 (301)	–0.36*	–14.04	15.60*
Summer	Personal $PM_{2.5}$ = ambient NO_2	24 (225)	0.42*	3.83	12.38*	15 (154)	0.38*	3.79	6.27*
Winter		45 (487)	0.24*	3.44	13.16*	30 (301)	0.26*	7.30	3.06*
Summer	Personal $PM_{2.5}$ = ambient CO	24 (225)	5.36	1.34	18.30*	15 (150)	1.87	0.50	13.42*
Winter		45 (487)	3.99*	3.17	15.00*	30 (301)	6.30*	10.97	3.24*
Winter	Personal $PM_{2.5}$ = ambient SO_2	45 (487)	–0.24*	–2.06	20.75*	30 (301)	–0.17*	–2.74	10.38*

*Significant at the 0.05 level.

Table 8. Association between personal $PM_{2.5}$ exposures and hourly maximum ambient O_3 and CO concentrations.

		Total personal $PM_{2.5}$ exposure				Personal exposure to $PM_{2.5}$ of ambient origin			
Season	Model	Subjects (n)	Slope	t-Value	Intercept	Subjects (n)	Slope	t-Value	Intercept
Summer	Personal $PM_{2.5}$ = ambient O_3	24 (225)	0.26*	6.22	4.33	15 (154)	0.27*	8.02	–3.66
Winter		45 (487)	–0.30*	–5.23	28.31*	30 (301)	–0.27*	–10.57	17.54*
Summer	Personal $PM_{2.5}$ = ambient CO	24 (225)	2.66	1.61	18.16*	15 (154)	–0.69	–0.40	14.94*
Winter		45 (487)	1.50*	2.64	15.94*	30 (301)	2.09*	7.97	5.12*

*Significant at the 0.05 level.

the associations between ambient pollutant concentrations and their respective personal exposures were essentially unbiased given the relatively high precision of the ambient pollutant monitors. As shown in Table 11 for the older adult cohort, the true significance of the models did not change, with all of the models remaining insignificant. For each model, estimates of both the true slope and true standard error increased, resulting in no appreciable difference in statistical significance. It should be noted that our ability to examine statistical significance may be limited by our relatively small sample size. With a larger sample size, it is possible that the corrected parameter estimates might become more statistically significant due to correcting the attenuation bias in the uncorrected estimates.

Discussion and Conclusions

For copollutants to be confounders of the epidemiologic associations between particles and adverse health effects, two conditions must be satisfied. They must be correlated with exposure to particles, and they must be correlated with the health outcome. We have shown that personal exposures to the gaseous air pollutants are not correlated, at least in our cohorts, with personal exposures to $PM_{2.5}$. Hence the gaseous copollutants cannot be confounders of $PM_{2.5}$ associations. Yet several studies have reported that ambient concentrations of gaseous air pollutants did confound observed associations between ambient particles and health. Why did this happen?

Ambient $PM_{2.5}$ concentrations were strongly associated with corresponding ambient concentrations of several gaseous copollutants in Baltimore, although the strength and direction of these associations differed by season. These results are consistent with findings from other studies and likely reflect common sources and meteorological conditions (*4,20*). Based on ambient results alone, therefore, it is possible that confounding by gaseous copollutants may impact observed associations between ambient $PM_{2.5}$ and adverse health.

With the exception of $PM_{2.5}$, however, ambient pollutant concentrations were weak indicators of their respective personal exposures. In many respects, these weak associations were not surprising given findings from earlier single-pollutant exposure studies that showed similarly strong longitudinal personal-ambient associations for particulate matter (*19,21,22*) and weak associations for the gases (*17,18,23,24*). For the gases, these weak associations can be attributed in part to low personal exposures, where personal exposures to O_3 and SO_2, in particular, were extremely low. Additionally, weak personal-ambient associations for the gases may be because variations in time spent outdoors, rather than variations in ambient concentrations, are the principal factor driving fluctuations in exposures to reactive gaseous pollutants over time. For a less reactive gas, such as NO_2, indoor sources may also weaken the association. This did not appear to affect the current results unduly, as similar results were shown for subjects living in residences with gas stoves as compared to electric stoves.

As could be expected from the previous pollutant relationships, the associations among the personal $PM_{2.5}$ and gaseous pollutant exposures were also weak and did not change in direction or significance when personal exposures to $PM_{2.5}$ of ambient origin were used in the analyses. These weak associations among personal $PM_{2.5}$, O_3, NO_2 and SO_2, together with the strong personal-ambient associations for $PM_{2.5}$, provide evidence that the observed $PM_{2.5}$-associated health effects are not due to confounding by the gaseous pollutants, at least for individuals with similar exposure profiles and living in similar urban locations. Additionally, differential sampler error, while present in varying amounts, accounted for at most 39% of overall exposure variability for the samplers used. This finding suggests that the reported associations were not unduly affected by reduced statistical power due to sampler error.

While exposures to the gaseous copollutants are unlikely to be potential confounders of $PM_{2.5}$, ambient copollutant concentrations were surrogates of personal $PM_{2.5}$. For all of the measured copollutants during both seasons, ambient copollutant

Table 9. Associations between ambient copollutant concentrations and personal exposure to $PM_{2.5}$ and its components for individuals with COPD, winter 1999.

Dependent variable	Independent variable	Slope	*t*-Value	*p*-Value
Ambient SO_4^{2-}	Ambient O_3			
Personal exposure to total $PM_{2.5}$	Ambient O_3	–0.25	–3.43	0.0008
Personal exposure to $PM_{2.5}$ of ambient origin	Ambient O_3	–0.27	–9.63	0.0001
Personal SO_4^{2-}	Ambient O_3	–0.02	–3.38	0.0009
Personal EC	Ambient O_3	–0.04	–5.34	0.0001
Ambient SO_4^{2-}	Ambient NO_2			
Personal exposure to total $PM_{2.5}$	Ambient NO_2	0.09	0.96	0.3376
Personal exposure to $PM_{2.5}$ of ambient origin	Ambient NO_2	0.29	8.3	0.0001
Personal SO_4^{2-}	Ambient NO_2	0.00	–0.09	0.9321
Personal EC	Ambient NO_2	0.05	5.06	0.0001
Ambient SO_4^{2-}	Ambient SO_2			
Personal exposure to total $PM_{2.5}$	Ambient SO_2	–0.20	–1.44	0.1524
Personal exposure to $PM_{2.5}$ of ambient origin	Ambient SO_2	–0.16	–2.49	0.0139
Personal SO_4^{2-}	Ambient SO_2	–0.03	–2.53	0.0125
Personal EC	Ambient SO_2	–0.01	–0.54	0.5927
Ambient SO_4^{2-}	Ambient CO			
Personal exposure to total $PM_{2.5}$	Ambient CO	1.36	0.88	0.3823
Personal exposure to $PM_{2.5}$ of ambient origin	Ambient CO	4.42	6.74	0.0001
Personal SO_4^{2-}	Ambient CO	–0.05	–0.32	0.7529
Personal EC	Ambient CO	1.02	6.38	0.0001

Table 10. Estimating the effects of sampler error.

Season	Pollutant	Personal COV (%)	Sampler error (%)	Percent of true variability[a]	Ambient COV (%)
Summer	$PM_{2.5}$	44	8	92	48
	O_3	104	9	91	25
	NO_2	81	14	86	27
Winter	$PM_{2.5}$	54	5	95	47
	O_3	566	9	91	57
	NO_2	73	39 (28)[b]	61	32
	SO_2	2,071	31	69	51

COV, coefficient of variation.
[a]Represents COV minus variability attributable to sampler error. [b]Indicates values after removing three outliers likely caused by filter contamination.

Table 11. Association between personal $PM_{2.5}$ exposures and personal copollutant exposures using slopes corrected for sampler error: models for older adults

Season	Personal $PM_{2.5}$ vs.	True slope	True SE	True *t*-value
Summer	Personal O_3	0.08	0.22	0.3
	Personal NO_2	0.24	0.09	2.6
Winter	Personal O_3	–0.29	0.36	–1.0
	Personal NO_2	–0.10	0.11	–1.4
	Personal SO_2	–0.85	0.93	–0.9

concentrations were shown to be better predictors of personal $PM_{2.5}$ than of their respective personal exposures. Associations involving personal $PM_{2.5}$ of ambient origin were even stronger. One-hour maximum ambient concentrations of O_3 and CO, which have also been associated with adverse health in epidemiologic studies, were similarly strongly correlated with personal exposures to both total $PM_{2.5}$ and that of ambient origin, indicating that the results were insensitive to the averaging time of these gaseous pollutants. In contrast, ambient $PM_{2.5}$ was a poor predictor of personal exposures to the gaseous copollutants. Together, these results demonstrate that the ambient concentrations of $PM_{2.5}$, O_3, NO_2, CO, and SO_2 are serving as surrogates for personal exposures to $PM_{2.5}$ alone.

Gaseous pollutants were stronger surrogates for $PM_{2.5}$ of ambient origin, as evidenced by the higher *t*-statistics for these comparisons. These stronger associations may be due to shared outdoor sources for the gaseous pollutants and $PM_{2.5}$ of ambient origin. Furthermore, some of the gaseous pollutants appear to be acting as surrogates for specific $PM_{2.5}$ components, as shown by the observed associations between ambient gaseous pollutant concentrations and personal EC and SO_4^{2-} exposures. For subjects with COPD, ambient CO and NO_2 were not significantly associated with total personal $PM_{2.5}$, but were associated with personal exposures to $PM_{2.5}$ of ambient origin and also to personal EC. These significant associations may be due to the fact that motor vehicles are a major source of CO, NO_2, EC, and, to a lesser degree, to $PM_{2.5}$ of ambient origin. Conversely, ambient CO and NO_2 were not significantly associated with personal SO_4^{2-}, a pollutant not associated with motor vehicle emissions. O_3, in contrast, was predominantly associated with personal SO_4^{2-}, an indicator of long-range transport and secondary particles.

The differences in significance among the cohorts may be attributable to differences in cohort-specific exposure patterns. For example, it is possible that although the total fraction of time spent outdoors was comparable, children spent more time outside during the peak O_3–$PM_{2.5}$ afternoon hours than older adults. This could account for the significance of the summertime association between personal O_3 and personal $PM_{2.5}$ for children but not for older adults. Observed cohort differences may also be due to differences in statistical power for each cohort.

If ambient copollutant concentrations are surrogates, as opposed to confounders, of $PM_{2.5}$, the results suggest that using multiple pollutant models in epidemiologic studies of $PM_{2.5}$ may not be suitable. As discussed by Breslow and Day (*25*), it is inappropriate to treat one variable as a confounder of another when both variables are actually surrogates of the same thing. In Baltimore, this would apply to epidemiologic models that incorporate ambient $PM_{2.5}$ as well as ambient O_3, NO_2, SO_2, or CO which have been shown in our analyses to be surrogates of personal $PM_{2.5}$. Depending on the strength of the true epidemiologic association, models that include these collinear, yet nonconfounding variables, will yield slopes for the causal pollutant factor ($PM_{2.5}$) that are underestimated (*5*). Likewise, the models will yield a misleading significant association for the collinear copollutant. Consequently, the correct modeling approach may be to exclude the gaseous pollutant concentrations for pollutants that are surrogates for particles rather than gaseous exposures and to employ single-pollutant regression models instead.

Additionally, results from this analysis clarify findings from epidemiologic studies. For example, in the recently published National Morbidity, Mortality, and Air Pollution Study (NMMAPS), data from 90 cities were compiled to assess the percentage change in mortality associated with changes in ambient air pollutant concentrations (*6*). The authors found that during the summer, increases of 10 ppb in ambient O_3 was associated with a 0.4% increase in mortality (95% CI; –0.20–1.01). Conversely, wintertime data indicated that the same increase in ambient O_3 led to a mean decrease of 1.86% in mortality (95% CI; –2.70–0.96), implying a protective effect from exposure to O_3. The peculiar wintertime results were described by the authors as "puzzling and may reflect some unmeasured confounding factor" (*6*). The results from the current analysis suggest that these results could be due to the fact that ambient O_3 is a surrogate for personal $PM_{2.5}$, where the observed negative wintertime associations between ambient O_3 and mortality reflect the negative association between ambient O_3 and corresponding personal $PM_{2.5}$.

Other recent studies have reported positive associations between ambient CO and respiratory hospital visits (*26*). Yet CO is neither a respiratory irritant nor a moderator of immune response in the respiratory tract, making those associations biologically implausible. $PM_{2.5}$, in contrast, has been shown to exacerbate respiratory infections (*27*) as well as produce an inflammatory response (*28*). The findings showing that ambient CO is a surrogate for personal $PM_{2.5}$ of ambient origin may, therefore, provide a biologically plausible explanation for the observed association between CO and respiratory hospital visits as well.

Our results were obtained in only one location, which is a limitation of this analysis. However, modulators of these associations between ambient concentrations and personal exposures, such as the amount of time spent outdoors and degree of ventilation in the home, were variable. Our sample included subjects who spent more time than average outdoors as well as subjects who spent less time than average outdoors. In addition, we had a wide range of indoor ventilation conditions in the homes sampled. We therefore believe that although different associations might be found in other cities, the qualitative results we report are unlikely to change.

In summary, the above results highlight the importance of properly characterizing associations among ambient pollutant concentrations and their personal exposures to air pollution epidemiologic studies. Studies conducted in locations with strong associations among ambient pollutant concentrations should not assume that associations observed among ambient pollutant concentrations necessarily persist among personal exposures to these pollutants, nor should they assume that relationships among ambient pollutant concentrations are consistent across seasons. In particular, ambient concentrations of gaseous air pollutants cannot be considered as surrogates for their respective personal exposures without site-specific evidence to support that assumption. Future research should focus on how specific factors, such as ventilation, time spent outdoors, and household characteristics, affect the strength of these associations for certain individuals and cohorts.

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Antiandrogenic Pesticides Disrupt Sexual Characteristics in the Adult Male Guppy (*Poecilia reticulata*)

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Environmental contaminants have been identified as endocrine disruptors through their antiandrogenic activity. Thus, as androgen receptor antagonists, the fungicide vinclozolin and the principal DDT metabolite *p,p′*-DDE have been demonstrated to induce demasculinization in rats. Whether this is also the case in fish remains to be demonstrated. For a period of 30 days, groups of adult male guppies were exposed to vinclozolin, *p,p′*-DDE, or the therapeutic antiandrogen flutamide (used as positive control) applied to the fodder at concentrations between 0.1 and 100 µg/g fodder. Subsequently, sexual characteristics of relevance to the male reproductive capacity were measured and compared with untreated control fish. All three chemicals caused profound alterations at increasing levels of biological organization, even in these fully matured males. At the cellular level, the three compounds induced a significant reduction in the number of ejaculated sperm cells. At the organ level, the sexually attractive orange-yellow coloration was reduced in area and discolored, and treated fish also had smaller testes. Further, at the organismal level, computer-aided behavior analyses demonstrated a severe disruption in male courtship behavior. We conclude that this demasculinization is consistent with an antiandrogenic action of vinclozolin and *p,p′*-DDE and is likely to compromise reproductive capability in this fish. *Key words*: antiandrogenic effects, courtship behavior, endocrine disruptor, flutamide, guppies, *p,p′*-DDE, *Poecilia reticulata*, sexual characteristics, vinclozolin. *Environ Health Perspect* 109:1063–1070 (2001). [Online _____]
http://ehpnet1.niehs.nih.gov/docs/2001/109p1063-1070baatrup/abstract.html

It is well documented that several chemicals from agricultural, industrial, and household sources possess endocrine-disrupting properties, which potentially pose a threat to human and wildlife reproduction (*1,2*). Most work has focused on the adverse effects of estrogenic substances (*3–5*). The discovery that certain environmental contaminants possess antiandrogenic properties (i.e., disrupt the normal function of the male sex hormones) has added to the endocrine disruption debate (*6,7*). The most intensively studied environmental antiandrogens are the dicarboximide fungicide vinclozolin and the insecticide DDT metabolite *p,p′*-DDE (*8–11*).

Extensive studies have demonstrated that vinclozolin and *p,p′*-DDE interfere with the action of androgens in developing, pubertal, and adult male rats (*10–15*). Exposure to vinclozolin and *p,p′*-DDE during the critical period of sexual differentiation results in sexual abnormalities expressed later in the adult male rat, including reduced anogenital distance, retained nipples, reduced sex accessory gland weights, urogenital malformations, and reduced fertility (*8,10,14,16–18*). With the same molecular mechanism and with almost the same potency as the classical antiandrogenic drug flutamide, both *p,p′*-DDE and the two primary vinclozolin metabolites, M1 and M2, bind the androgen receptor (AR) and act as antagonists by preventing transcription of androgen-dependent genes (*9,11,13,18*). Androgen-induced gene products play a key role in the development and maintenance of male sexual functions, including courtship behavior (*19*) and spermatogenesis (*20*).

The potential threat of environmental antiandrogens to fish and wildlife has been addressed by Monosson et al. (*21*). Although the authors noted that the antiandrogenic activity of *p,p′*-DDE is unknown in nonmammalian species, they suggested that this property may have contributed to the reproductive abnormalities in the American alligators in Lake Apopka (*22,23*) and the near absence of male bloaters in Lake Michigan in the late 1960s.

Androgen receptors have been characterized in a few fish species. Sperry and Thomas (*24,25*) identified two distinct androgen receptors, AR1 and AR2, in brain and gonadal tissues of kelp bass (*Paralabrax clathratus*) and Atlantic croaker (*Micropogonias undulatus*) with different tissue distributions and distinct steroid and xenobiotic-binding specificities. AR1 was found to bind only testosterone with high affinity, but AR2 bound a broader range of natural androgens and antiandrogens, including *p,p′*-DDE and the vinclozolin metabolites M1 and M2. In particular, M2 binds AR2 in both testicular and ovarian tissue with an affinity nearly identical to the AR in rats. Wells and Van der Kraak (*26*) found a single class of high-affinity, low-capacity AR in rainbow trout (*Oncorhynchus mykiss*) brains and in ovaries, testes, and brains of goldfish (*Carassius auratus*). This study suggested a relatively high affinity between *p,p′*-DDE and the goldfish testes AR, whereas *p,p′*-DDE, M1, and M2 showed no significant competition for the AR in any of the remaining tissues tested in the two fish. Likewise, vinclozolin, M1, and M2 failed to compete for high-affinity testosterone binding sites (putative androgen receptors) in the fathead minnow, *Pimephales promelas* (*27*). Accordingly, as pointed out by Sperry and Thomas (*25*), multiple androgen receptor subtypes may be present throughout teleost species and target tissues, with differential affinities to natural androgens and different susceptibilities to xenobiotic interference.

Endocrine-disrupting chemicals (EDCs) are believed to propagate their initial molecular interactions to higher level effects in the endocrine system and reproductive organs, ultimately resulting in an impaired reproductive capability. Thus, disruption of hormonal functions can be expressed at various levels of the vertebrate endocrine system (*28*). Molecular markers (e.g., vitellogenin synthesis and AR binding studies) can be highly sensitive to demonstrate the presence of EDCs in the environment, but the vertebrate endocrine system is so complex that it is impossible to predict higher level effects solely from events at the receptor level. For that purpose it is necessary to identify end points that are more directly related to the reproductive fitness of the individual and preferably with links to population-level effects. We have addressed this objective in a series of laboratory experiments for the purpose of studying the effects of EDCs on selected sexual characteristics in the guppy (*Poecilia reticulata*).

The guppy was chosen as an experimental animal because it is a viviparous fish which breeds year round and has a short reproductive period (*29*). The adult male has a bright orange coloration and performs a distinct courtship behavior. His anal fin is developed into a copulatory organ (the gonopodium) for internal fertilization, and ejaculates of sperm can be evacuated for sperm counting

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We thank F. Barlach for the elegant software development.

This work was supported by the Danish Environmental Research Program and the Danish Environmental Protection Agency.

Received 8 January 2001; accepted 6 April 2001.

without harming the fish (*30,31*). The hormonal pathways controlling the expression of the male sexual characteristics are not fully understood, but sperm production, body coloration, and courtship behavior are known to be regulated by androgens (*32–34*). Hence, the male guppy offers a suite of sexual characteristics that are both accessible for quantification and of relevance for the study of EDCs on reproduction. In this study, adult male guppies were exposed for 30 days to vinclozolin, *p,p′*-DDE, and the therapeutic antiandrogen flutamide, administered in the food. Subsequently, we assessed the effects of these three chemicals on the number of ejaculated sperm cells at the cellular level, body coloration, length of gonopodium (copulatory organ), and relative gonadal weight at the organ level, and finally courtship behavior at the organismal level. Previous studies have demonstrated that most of these end points are altered in adult male guppies exposed to the natural estrogen 17β-estradiol and the xenoestrogen 4-*tert*-octylphenol (*31,35*).

Material and Methods

Animals and experimental conditions. The fish used in this study were healthy, wild-type guppies (*Poecilia reticulata*) imported from Colombia and bred through several generations in 500-L stainless-steel tanks at 25 ± 2°C and a daily 12-hr simulated daylight illumination. These stock aquaria received fully aerated water from a reverse osmosis system (RO-water), which was mixed with local tap water (9:1) and adjusted with NaCl to give a conductivity of 600 µS/cm and a pH of 7.0 ± 0.3. Half of the water in the aquaria was renewed weekly. The guppies were fed daily with freshly hatched *Arthemia sp.* and commercial flake food (TetraMinRubin and TetraMin, Tetra Werke, Melle, Germany).

A total of 260 adult males were chosen randomly from the stock aquarium and divided into 10 experimental groups and 3 control groups. Each group was transferred to a 16-L seamless glass aquarium (Struers Kebo Laboratory, Copenhagen, Denmark) filled with 4 L of RO-water and 4 L of water from the culture tank. During the experimental period of 30 days, the water was constantly circulated through a natural filter of aquarium gravel. Daily, feces were removed and clean RO-water was added to 8 L. To eliminate the risk of leached EDCs, no plastic materials or plants were used in any aquaria and plumbing.

The fish were exposed for 30 days through their food to one of the three antiandrogens: the dicarboximid fungicide vinclozolin [3-(3,5-dichlorophenyl)-5-methyl-5-vinyloxazolidine-2,4-dione], *p,p′*-DDE (*p,p′*-1,1-dichloro-2,2-bis (*p*-chlorophenyl) ethylene (both chemicals from Riedel-de-Haën AG, Seelze, Germany), and the commercial antiandrogen flutamide (4′-nitro-3′-trifluoromethylisobutyranilide; Sigma Chemicals, St. Louis, MO, USA). Flutamide is a specific inhibitor of the androgen receptor and was therefore used as the positive control of antiandrogenic effects. All three chemicals were dissolved in acetone to adequate concentrations, mixed thoroughly with the commercial TetraMin fish fodder and left for 24 hr in a fume cupboard for the evacuation of acetone. This resulted in fodder contaminated with 1.0, 10.0, and 100.0 µg vinclozolin or flutamide per milligram fodder and 0.1, 1.0 or 10.0 µg *p,p′*-DDE per milligram fodder. The remaining three control groups received food that was mixed with acetone only. Each group of 20 fish was fed daily with 40 mg fodder, corresponding to 0.2, 2, 20, or 200 µg chemical per fish. Assuming an equal consumption of food by the fish and that the average weight of an adult male guppy is 130 mg, the fish at the three application rates of vinclozolin or flutamide were dosed with 15, 150, and 1,500 µg chemical/g fish and fish were dosed with *p,p′*-DDE at 1.5, 15, 150 µg/g fish.

After exposure, we measured male sexual characteristics of importance to guppy reproduction and expected targets of antiandrogenic action at increasing levels of biological organization. The number of ejaculated sperm cells were measured at the cellular level, body coloration, length of gonopodium (copulatory organ), and testis size (gonodosomatic index) at the organ level, and courtship behavior at the organismal level.

Sperm count. Immediately after behavior analysis, the male was lightly anesthetized in ethyl-4-aminobenzoate (Sigma) and placed on a glass plate under an Olympus SZ 40 dissection microscope mounted with a circular illumination of polarized light and a JVC TK-1070E color video camera (Victor Company of Japan LTD, Tokyo, Japan). The gonopodium was swung forward and a 32-bit 1,024 × 1,024 pixel digital image of the fish's left side was captured by a VISTA frame grabber (TRUEVISION, Santa Clara, CA, USA) and stored on disk for later measurements of gonopodial length and coloration. Sperm cells were stripped from the male guppy by gently stroking the abdomen with a small metal rod toward the gonopodium, thereby evacuating an ejaculate on the glass plate. The guppy ejaculate consists of numerous spermatozeugmata (clusters of sperm cells), which were collected with a Finn-pipette and transferred to 90 µL of a 0.125 mM NaCl and 5.0 mM $CaCl_2$ solution to aid the breakdown of the spermatozeugmata. The pipette was filled and emptied 30–40 times to ensured the final disintegration of the spermatozeugmata. Samples of the sperm cell suspension were then transferred to an improved Neubauer chamber hemacytometer (Paul Marienfeld, Bad Mergentheim, Germany) and, after 10 min retention in a humid chamber, counted using the general guidelines for human sperm (*39*). This method gives a measure of the total number of sperm cells in an ejaculate. This method has high reproducibility in individual guppies over time. Toft and Baatrup (*31*) showed, using this method, that the sperm count in uncontaminated guppies remained constant when sampled at time 0, day 30, and day 90.

Gonadosomatic, coloration, and gonopodial indices. The fish was killed in ethyl-4-aminobenzoate and fixed in Lilly's formalin solution. We determined and calculated the wet weights of whole body and testis and calculated the gonadosomatic index (GSI) as the gonadal weight as percentage of the whole-body weight.

The total area of the orange-colored spots was measured in the digital image of the fish and related to the whole body area (fins excluded) as the coloration index. Hereafter, the length of the gonopodium was measured and related to the length of the fish as the gonopodial index. Digital image analyses were performed using GIPS software (Image House, Copenhagen, Denmark).

Male courtship behavior. Sexually mature guppies perform courtship behavior almost continuously during the light hours, all year round. Guppy sexual behavior has been described thoroughly (*36,37*). Briefly, the male places himself in front of the female and stays within her field of view (posturing behavior). From this position he performs the sexual display toward the female known as sigmoid display, where his body assumes the shape of an "S" or "C" (hence the name of this behavior), and vibrates while he swims sideways displaying his sexually attractive orange-yellow coloration. He either moves along the length of the female to come into position for a copulation, or he moves away from the female, remaining in her field of view to entice her to follow. These behavioral maneuvers are so stereotyped and performed so frequently (about 1/min), even in a laboratory setting, that the male guppy's courtship behavior lends itself to quantification. This makes the guppy and its sexual behavior a suitable biomarker of endocrine disruption (*35*).

Analysis of courtship behavior. Guppy courtship behavior was measured automatically using the newly developed computer-aided DISPLAY vision system (Institute of Biological Sciences, University of Aarhus, Denmark), which records and analyzes complex behavior patterns in fish.

After exposure, each male was paired with a 4-month-old, nonreceptive female in a sand-blown 20 × 15 cm aquarium containing 1.8 L of 25°C water (water depth, 10 cm) placed on a sheet of glass 50 cm above diffusely-lit white paper. We used nonreceptive females to preclude copulations and hence to ensure constant female responses toward all males. The entire setup was enclosed in a metal frame covered with a blackout curtain. When viewed from above, this arrangement resulted in clear silhouettes of the two fish, where the male was easily distinguishable from the much larger female (Figure 1). The pairs were left undisturbed for 5 min, after which the scenario was recorded for 10 min.

A CV-M10 progressive scan (non-interlaced) CCD camera (JAI, Inc., Copenhagen, Denmark) mounted 50 cm above the aquarium displayed an image of the aquarium on a monitor. Simultaneously, the analogue video signal from the camera was digitized by a DT3155 frame grabber (Data Translation, Inc., Marlboro, MA, USA) into a 768 × 576-pixel digital image, giving a 0.25-mm spatial resolution of the visual field. The frame grabber was interfaced with a 300-MHZ Pentium II personal computer.

Prior to recording, the interior of the aquarium was framed by a software window (region of interest), and appropriate size and gray-level ranges corresponding to the fish silhouettes were likewise set in the software. These criteria were used for the conversion of each 8-bit gray-scale image into a binary (1-bit) image. Thus, all pixel assemblages fulfilling both size and gray-level criteria (fish silhouettes) were assigned the value 1, while the remaining pixels in the image were given the value 0. This new binary image was stored in a frame file on disk for subsequent analysis. During recording, an image was captured and processed approximately every 1/12 sec, so each 10-min frame file contained about 7,200 binary frames (occupying only about 6 MB disk space).

The frame files, containing the time-series of fish contours, were subsequently analyzed by the DISPLAY program. An exhaustive description of this software is beyond the scope of this paper, but the most important steps in the characterization and quantification of the complex courtship behavior are outlined below.

First, the position and orientation of the two fish within the digital image (global coordinate system) must be established in each frame. The two oblong pixel assemblages, representing the fish silhouettes, are converted into two small coordinate systems by determining their principal axes. The axes can be found by computing the eigenvectors (*38*) for the position vectors of the pixels. The mean pixel position vector is the origin of the coordinate system spanned by the eigenvectors. The eigenvectors constitute a unitary transformation which enables transformation of other coordinate systems into a particular coordinate system, such as the other fish's coordinate system or the global coordinate system. Thus, all angle and distance measures can easily be computed once the principal axes of the pixel masses are determined. In order to associate a directed coordinate system to the pixel mass, the number of object-pixels inside two circles of equal radius are counted two standard deviations from the origin along the *y*-axis in both directions. The direction with the largest pixel count gives the direction of the fish's head. Furthermore, object shape measures can be computed by dividing the object pixel mass into parts. In this case, the parts are simply positive and negative (above and below the *x*-axis) position vectors of the object pixels. Each part is subsequently treated as a new object, and the angle between the new *y*-axes provides a measure of the fish curvature (Figure 1C).

For each frame it is now possible to determine the position and orientation of each fish relative to the other, the distance between them, and the curvature of the male. Further, frame-to-frame comparisons enable calculation of speed and direction (relative to the body's longitudinal axis) of fish movements. The composite courtship behavior of the guppy, including posturing behavior and sigmoid displays, can be broken down into its constituting elements, including the mutual position/orientation of the two fish and their movement patterns. The following parameters were extracted from each of the approximately 7,200 frames in the frame file: *a*) position of female relative to the male measured as the angle (0–180°) between the male's *y*-axis and a line between the origins of the two fish's coordinate systems (centroids; ∡ε in Figure 1A); *b*) position of male relative to the female measured as the angle (0–180°) between the female's *y*-axis and a line between the centroids of the two fish (∡δ in Figure 1A); *c*) distance between the centroids of the two fish (*a* in Figure 1A); *d*) male swimming speed defined as the frame-to-frame displacement of his centroid divided by the time between successive frames (*v* in Figure 1B); *e*) male angular displacement measured as the angle (0–180°) between the male *y*-axis and the position vector of his centroid in the next frame (∡φ in Figure 1B), where high values indicate sideways and backward swimming; *f*) male lateral velocity calculated as the magnitude of the male velocity component perpendicular to the *y*-axis in the preceding frame (*u* in Figure 1B), signifying the intensity of sideways swimming; and *g*) male curvature measured as the angle between the *y*-axes of head region and the tail region, respectively (∡ω in Figure 1C).

The frame-to-frame measurements of the seven parameters were subsequently used to identify periods with posturing behavior and sigmoid displays within the entire 10-min frame file. This was done by assigning a range of pass values (search criterion) to each parameter. Basically, a specific behavior is recognized when all search criteria are fulfilled simultaneously. Thus, before analyzing the frame files, the appropriate combination of search criteria describing the specific behavior is set once for all. This is done by repeatedly refining and verifying the search

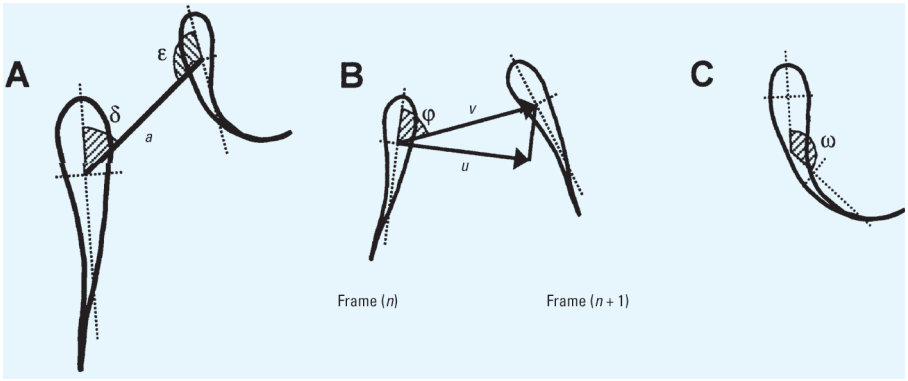

Figure 1. Composite courtship behavior of the male guppy automatically quantified by the DISPLAY vision system using seven descriptive components. Abbreviations: *a*, distance between female and male; *u*, male lateral velocity measured as the velocity component perpendicular to the longitudinal axis of the male; *v*, male swimming velocity; ∡δ, angular position of the male relative to the female's longitudinal axis; ∡ε, angular position of the female relative to the male's longitudinal axis; ∡φ, male angular displacement relative to his longitudinal axis; ∡ω, male curvature measured as angle between the y-axes of head region and tail region. (*A*) The two oblong pixel assemblages representing the male and female silhouettes from the digital image were converted into directed coordinate systems, which enabled the calculation of position and orientation of each fish relative to the other and the distance between them. (*B*) Frame-to-frame comparisons enabled calculations of speed and direction (relative to the body's longitudinal axis) of fish movements. (*C*) The body curvature was measured as the angle between the two y-axes of coordinate systems aligned with the head and tail regions, respectively.

criteria until the software's interpretation of the behavior in all situations agrees with this particular behavior. This manipulation is easily performed by combining a graphical user interface for entering search criteria with a real-time replay facility in the DISPLAY program.

The measurement of the male's posturing behavior (i.e., the time he spent in front of the female introducing the next sigmoid display) involved three search criteria. First, the male must be in front of the female (pass values of ∡δ in Figure 1A set to $0 < \delta < 90°$). Second, the male must at least partly face the female (∡ε criterion in Figure 1A set to $0 < \varepsilon < 60°$). Note that all angles are presented without signs since there is no distinction between the right side and left side of the fish. Finally, the two fish must be within a distance of a few centimeters (*a* in Figure 1A set to < 60 mm).

The guppy sigmoid display is a much more complex behavior. First, the combination of involved parameters and their ranges of pass values changes during the course of the display. Accordingly, a positive identification of the entire display by the software requires temporal adjustments of the search criteria. This was achieved by associating a timer to each search criterion, engaging and disengaging its function. The onset of the display is characterized by the male being within the anterior part of the female's field of view for at least 0.2 sec ($0 < \delta < 90°$; time out 0.2 sec) at a distance of at least 22 mm ($a > 22$ mm) exposing the side of his body ($\varepsilon > 68°$), which is locked in a distinct curvature for at least 0.5 sec ($\omega > 20°$; time out 0.5 sec). After this initial phase the rules are changed allowing the male to move within the female's entire field of view ($0 < \delta < 140°$), but with his body still locked in a curvature ($\omega > 5°$) with the convex side continuously facing her ($\varepsilon > 68°$). Display termination is registered when one or more of these criteria are no longer fulfilled.

The frame files of all treated and control fish were analyzed by the DISPLAY software. The complete analysis of a 10-min frame file takes about 25 sec with a 300 MHz Pentium II computer. The time devoted to positioning behavior, number, and duration of the sigmoid displays and the average values of the measured parameters were saved in a data file for subsequent statistical analysis.

Statistical analyses. Where necessary, data sets were transformed to comply with the normality and variance homogeneity requirements for analysis of variance (ANOVA) testing to compare means among the treatment groups. Subsequently, Dunnett's test for multiple comparisons was used to determine whether treatment means were significantly different ($p < 0.05$) from the control group. All statistical tests were performed with SPSS software (SPSS for Windows, release 9.0; SPSS Inc., Chicago, Ill, USA).

Results

In the group fed the highest concentration of vinclozolin (100 µg/mg), 15% of the fish died during the 30 days of treatment. The corresponding mortalities with the highest concentrations of DDE (10 µg/mg) and flutamide (100 µg/mg) were 70% and 35%, respectively. Because the chemicals were obviously toxic at these application rates, these three groups were excluded from further evaluation of antiandrogenic effects. In contrast, at the 10 and 100 times lower application rates, none of the fish displayed obvious toxic responses such as body darkening or changed swimming activity. The few fish that were lost in these groups died solely because of incorrect handling.

All three chemicals caused pronounced effects on the adult male's sperm count, body coloration, testes size, and courtship behavior. Below, the effects on these sexual characteristics are presented in the order they were measured.

Sperm count. The provoked ejaculate from control fish contained an average 5 million sperm cells. All antiandrogen treatments reduced this number, except in the group fed *p,p′*-DDE at 0.1 µg/mg, where the sperm count actually exceeded that of control fish (Figure 2B). The lowest sperm count, at 1.6 million sperm cells, was measured in the 10 µg/mg flutamide group. The ejaculates of the remaining groups contained 3–4 million sperm cells.

Coloration and gonopodial indices. The demasculinizing effects of the antiandrogens also influenced the area and color intensity of the male orange coloration. In the control group, an average 12% of the body surface (coloration index) was coved with orange spots, while this percentage was lower in the treated groups (Figure 2C). Statistically, the reduction in coloration index was only significantly different from the control fish in the group treated with vinclozolin at 1 µg/mg. Even with the naked eye it was obvious that the treatments also caused discoloring of these sexually attractive spots. Measurements of the red, green, and blue color components in the digital images demonstrated that this fading was primarily caused by a significant brightening of the blue component in all treated groups, with the exception of the low dose *p,p′*-DDE group (data not shown).

The length of the gonopodium relative to the length of the fish (gonopodial index) was unaffected by the antiandrogens, as was the size of the fish.

Gonadosomatic index. The weight of the testis relative to the body weight (gonadosomatic index) was significantly lower in the fish exposed to antiandrogens with the exception of the group treated with the low *p,p′*-DDE dose (Figure 2D). In the control

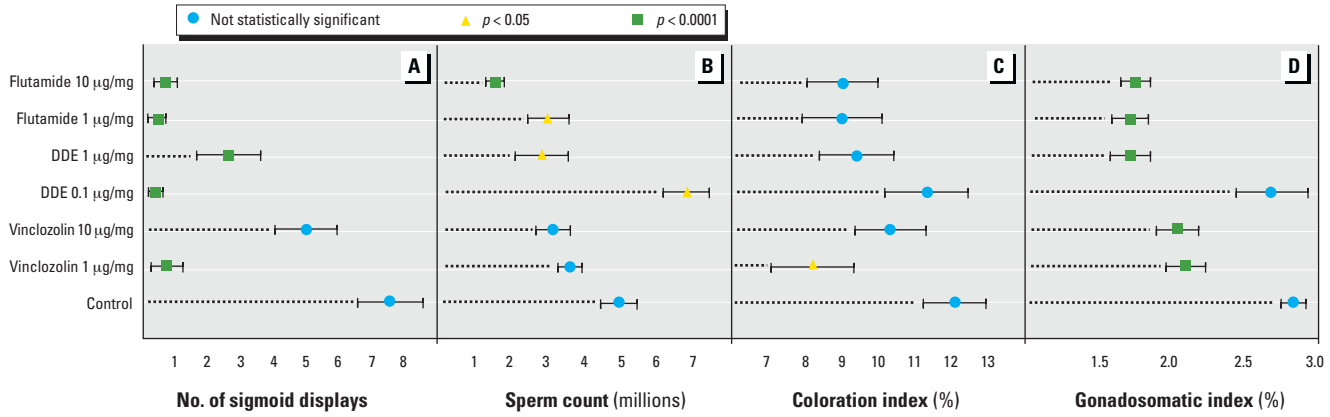

Figure 2. The effects of a 30-day exposure to vinclozolin, *p,p′*-DDE, and flutamide on four sexual characteristics of the adult male guppy. (*A*) Number of sigmoid displays during the 10-min recording period. (*B*) Number of sperm cells in a provoked ejaculate. (*C*) Area of orange coloration as percentage of body area (coloration index). (*D*) Weight of testis as a percentage of whole-body weight (gonadosomatic index). Statistical differences between the control group and the treated groups were tested with one-way ANOVA followed by Dunnett's post-hoc multiple comparisons test.

group, the testes made up about 2.8% of the body weight, whereas GSI values between 1.7 and 2.0% characterized fish treated with the three chemicals.

Courtship behavior. Of the seven behavioral elements (Figure 1) measured by the automated vision system, four were used in combinations to quantify the two most important behavioral patterns in the courtship behavior—namely, the posturing behavior and the sigmoid display. The accuracy by which the vision system identified these two composite behavior patterns was assessed by replaying the frame sequences. All situations with posturing behavior were correctly quantified by the system, and of 464 sigmoid displays identified in the 145 recordings, only 24 cases were considered questionable by two independent observers and therefore excluded from further analysis.

The effects of vinclozolin, *p,p′*-DDE, and flutamide on each of the seven behavioral elements are presented as average values in Table 1. Considered individually, two behavioral components of the sexually active male guppies were particularly affected by the three compounds. The males from the treated groups were less oriented toward the female (∡ε in Table 1) and swam less sideways, both as regards swimming direction (∡φ in Table 1) and sideways swimming velocity (*u* in Table 1). The male's efforts to face the female were most strongly restrained by vinclozolin at 1.0 µg/mg fodder, whereas *p,p′*-DDE most effectively impeded the sideways swimming activity. Surprisingly, some of the behavioral elements displayed a negative or neutral dose–response relationship. For instance, the inhibition of male orientation toward the female and his sideways swimming was more pronounced in the group that was treated with 1 µg vinclozolin/mg fodder than in the group treated with the 10 times higher concentration. The same two behavioral components were affected to the same degree by the two DDE concentrations. The remaining elements were less influenced by the antiandrogenic treatments, including the male's position relative to the female (∡δ in Table 1), his body curvature (∡ω in Table 1), and the distance between the two fish (a in Table 1). It is worth noting that there was no statistical difference in the general swimming velocity (*v* in Table 1) between the males in the treated groups and the control group. Significant differences in average swimming velocity could indicate a general toxic effect of the chemicals.

The two composite patterns in the male guppy's courtship behavior, the posturing behavior and the sigmoid display, were more strongly affected by the three antiandrogens than any of the separate constituent components. In all cases, the chemicals weakened the male's sexual activity. With the face-to-face posturing behavior, the male tried to attract the female's attention before he performed the sigmoid display. The influence of the three antiandrogens on the duration of posturing behavior within the 10 min of recording is shown in Figure 3. The males from the control group spent on average 130 sec in posturing behavior, corresponding to 22% of the observation period. The most serious inhibition of this behavior was found in the group fed vinclozolin at 1 µg/mg fodder, where the males on average fulfilled the criteria of posturing behavior for only 40 sec, or 7% of the recording period. In comparison, the group treated with flutamide at the same concentration spent about 15% of the time on posturing. The composite behaviors also demonstrated neutral or negative dose–response relationships. Hence, in the group treated with vinclozolin concentration at 10 µg/mg fodder, the posturing behavior was unaffected, whereas flutamide at this concentration inhibited this behavior by 51% relative to the controls. Correspondingly, *p,p′*-DDE restrained the posturing behavior by 20% and 43% at 0.1 and 1.0 µg/mg, respectively, when compared with the control group.

Although the sigmoid display is the most conspicuous movement pattern in the male guppy's courtship behavior, it makes up only a small part of the courtship temporally. The total duration of the male's mating behavior, including both posturing behavior and sigmoid displays, is presented in Figure 3. As a prelude to the copulation attempt itself, the sigmoid display is the culmination of the courtship behavior. The number of sigmoid displays is therefore a suitable measure of the male's mating ardor. Figure 2A demonstrates that males from the control group performed on average about eight sigmoid displays per 10 min observation period, whereas this behavior only rarely occurred in the groups treated with flutamide or the lowest concentrations of vinclozolin (1.0 µg/mg) and *p,p′*-DDE (0.1 µg/mg). This component in the courtship behavior also demonstrated a negative dose–response relationship, with much less inhibition at the high application rates of the two pesticides.

Discussion

Oral administration of either vinclozolin, *p,p′*-DDE, or flutamide clearly altered the sexual characteristics of the adult male guppy. After only 30 days of exposure, the orange display coloration was reduced in both area and color

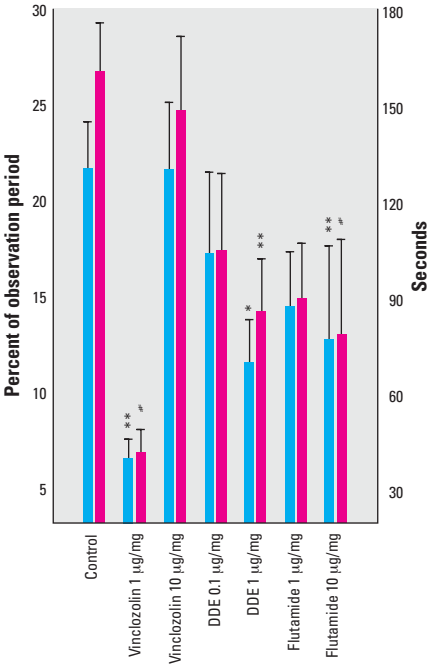

Figure 3. The three antiandrogens, vinclozolin, *p,p′*-DDE, and flutamide, reduced the time allocated to courtship behavior in the adult male guppy. Blue bars indicate the average duration of posturing behavior during the 10-min recording period. Red bars show the total time devoted to both posturing behavior and sigmoid displays.
**p* < 0.05, ***p* < 0 .001, and #*p* < 0.0001 as determined by Dunnett's post-hoc test.

Table 1. The effects of vinclozolin, *p,p′*-DDE, and flutamide on seven components in the courtship behavior of the male guppy.

Treatment (µg/mg fodder)	No.	∡ε (degrees)	∡δ (degrees)	*a* (mm)	*v* (mm/sec)	∡φ (degrees)	*u* (mm/sec)	∡ω (degrees)
Control	(19)	44.1 ± 4.3	84.6 ± 5.8	54.9 ± 3.4	17.4 ± 1.5	69.6 ± 2.8	7.4 ± 0.7	9.4 ± 0.4
Vinclozolin, 1.0	(18)	82.5 ± 4.4#	80.2 ± 4.7	75.2 ± 6.8*	20.4 ± 2.5	46.2 ± 3.0#	4.5 ± 0.6**	8.5 ± 0.6
Vinclozolin, 10.0	(18)	60.7 ± 5.5	75.8 ± 3.2	53.9 ± 3.5	18.8 ± 1.8	59.7 ± 5.3	5.2 ± 0.5**	7.5 ± 0.4
DDE 0.1	(17)	69.2 ± 6.6**	81.5 ± 4.1	58.1 ± 6.5	13.2 ± 2.2	59.6 ± 4.8	3.1 ± 0.6#	6.4 ± 0.6#
DDE 1.0	(16)	68.0 ± 5.3**	75.8 ± 3.5	68.4 ± 6.2	15.3 ± 2.1	51.6 ± 6.0*	3.5 ± 0.4#	7.1 ± 0.6
Flutaminde,1.0	(17)	71.1 ± 5.3**	79.8 ± 3.5	67.6 ± 6.5	16.0 ± 1.9	52.4 ± 3.7**	4.6 ± 0.6**	8.5 ± 0.7
Flutaminde, 10.0	(18)	78.7 ± 5.8#	77.3 ± 4.7	91.8 ± 8.7#	19.6 ± 1.8	39.6 ± 3.4#	4.6 ± 0.5#	7.2 ± 0.3#

Differences between treated groups and control group were tested with one-way ANOVA followed by Dunnett's post-hoc multiple comparisons.
**p* < 0.05; ***p* < 0.001; #*p* < 0.0001.

intensity, the weight of the testis was diminished, the sperm count had fallen, and the courtship behavior was almost extinguished. To our knowledge, the present study provides the first evidence that these pesticides can cause severe reproductive abnormalities in fish.

Two previous studies, involving *p,p′*-DDE and vinclozolin concluded that there was no evidence that these two chemicals act as endocrine disruptors in fish. Carlson et al. (*40*) microinjected embryos of rainbow trout (*Oncorhynchus mykiss*) and chinook salmon (*Oncorhynchus tshawytscha*) with a number of contaminants, including *p,p′*-DDE. After rearing for 6 months, no treatment-dependent changes in sex ratio, gonadal histology, or steroid production were observed. Similarly, embryonic fathead minnows (*Pimerhales promelas*) were exposed to vinclozolin in the water by Makynen et al. (*27*), who concluded that vinclozolin had no adverse effects with respect to sexual differentiation and reproductive success despite the fact that data showed a 20–60% reduction in fecundity. In contrast, exposure of adult fathead minnows to vinclozolin resulted in increased plasma 17β-estradiol in males and a decline in the gonadosomatic index of females accompanied by a retardation in oocyte development (*27*). Accordingly, the three studies performed till now reached different conclusions regarding the endocrine-disrupting properties of vinclozolin and *p,p′*-DDE. These apparent discrepancies may be explained by differences in chemical concentrations, route of application and time-window of exposure, but more likely reflect real species differences in the sensitivity of the reproductive apparatus to antiandrogenic compounds. Considering that fish display a wide range of reproductive strategies and the growing evidence of interspecies and tissue differences in AR binding specificity, some fish may be more susceptible than others to endocrine disruption by a particular chemical (*21,25,26*).

The three chemicals used in this study caused impairment of the guppy's sexual characteristics in a manner consistent with the effects of antiandrogens. First, the affected sex characteristics are known to be under androgen control in the guppy (*32,41–43*). Thus, Pandey (*32*) blocked the synthesis of sex steroids in adult male guppies by hypophysectomy, which caused a marked regression in the testis, inhibition of spermatogenesis, and a pronounced fading of the orange display coloration. Subsequent treatment of these hypophysectomized males with the androgen methyl testosterone induced partial restoration of coloration and testis morphology and function (*42*). Similarly, it has been shown that the guppy's sexual behavior is under androgen control (*37,41,44*), as is the case with other fish species (*33*). Accordingly, the changes in the sexual characteristics induced by vinclozolin and *p,p′*-DDE closely parallel those evoked by androgen deprivation. Second, it has been thoroughly established that both vinclozolin and *p,p′*-DDE are functional antiandrogens in mammals by blocking the androgen receptor (*9,10,12,18*) in the same way as the therapeutic drug flutamide, which acts purely as an antiandrogen (*45,46*). Collectively, these considerations strongly suggest that vinclozolin and *p,p′*-DDE act as endocrine disruptors in the guppy by antagonizing the androgen receptor. The altered sexual characteristics represent a significant reduction in the expression of the male phenotype, indicating that the reproductive fitness of antiandrogen treated fish was impaired.

An appropriate sexual behavior is prerequisite for mating success in most animals. Unbiased measures of the guppy courtship behavior were obtained using the newly developed vision system, designed to identify complex behavioral patterns in fish. The sexual instinct of the male guppy was seriously compromised by vinclozolin, *p,p′*-DDE, and flutamide, which significantly reduced the time devoted to posturing behavior and almost eliminated sigmoid displays. It has been demonstrated that males with a high sigmoid display frequency are preferred by females (*47,48*) and that male mating success is positively correlated to the intensity of sigmoid displays (*47,49,50*). Also, Matthews et al. (*30*) found a strong correlation between display rate and sperm number, hence providing further evidence of the link between sexual behavior and reproductive capacity in male guppies.

The male orange coloration is similarly thought to signal condition and genetic quality. Several studies have shown that male guppies with the largest and brightest orange spots are favored by females and that these males have a higher mating success (*48,51,52*). Impairment of the male coloration is therefore likely to reduce reproductive fitness. In addition to antiandrogens, discoloration of the orange spots has been reported in response to other chemical and natural stressors, including estrogen (*31,53*), the xenoestrogen octylphenol (*31*), food quality (*50*), and parasites (*54*).

The relationship between sperm count and Darwinian fitness is less clear. Kime (*55*) noted that it is difficult to relate sperm count in fish to population-level effects because the amount of ejaculate necessary for successful fertilization is unknown. However, Warner (*56*) has argued that most male fish release only the minimum amount of sperm that is required for fertilization, so that any decrease in sperm quantity or quality will result in reduced fertility. In this study, the number of sperm cells in the provoked ejaculates of the treated groups was reduced 20–60% when compared with the control group. This reduction in sperm count may be a simple consequence of the diminished testis size in the exposed fish and/or caused by a direct antiandrogenic action of the chemicals on spermatogenesis. Inhibited spermatogenesis in response to antiandrogenic compounds has been reported in a number of vertebrates, including fish (*57*), amphibia (*58*), hamsters (*59*), and humans (*60*). In particular, significant reductions in epididymal sperm counts have been demonstrated in rats treated with *p,p′*-DDE (*61*) and vinclozolin (*18*). In contrast, Moorman et al. (*62*) found a surprising increase in sperm counts in sexually mature rabbits after dermal application of vinclozolin during the peripubertal period. Still, inhibited spermatogenesis appears to be the general rule of antiandrogenic exposure.

The gonopodium was unaffected by exposure to vinclozolin, *p,p′*-DDE, or flutamide. This is in agreement with Pandey (*42*), who found that the morphology of the gonopodium in adults was insensitive to steroid depletion by hypophysectomy and concluded that once morphogenesis of skeletal elements is completed, it becomes independent of the pituitary hormones and androgens. The development of the gonopodium is certainly under androgen control because adult female guppies fed 17α-methyltestosterone developed gonopodia (*44*). Also, a parallel study in our laboratory has demonstrated that significantly smaller gonopodia evolved in guppies treated with the three antiandrogens during juvenile development (*63*).

Overall, the three antiandrogens affected the selected sexual characteristics in the same direction. Identical amounts of fodder were added to all aquaria daily, and all fodder was consumed before feeding the following day. The fish appeared eager to feed in all treatments and throughout the experiment, so we could not detect any possible differences in palatability between chemicals. Considering the molar concentrations of the three chemicals in the fodder (vinclozolin: 3.5 and 35 µmol/g fodder; *p,p′*-DDE: 0.31 and 3.1 µmol/g fodder; flutamide: 3.6 and 36 µmol/g fodder), it appears that the antiandrogenic potencies *in vivo* of vinclozolin and *p,p′*-DDE equaled, and in some cases even exceeded, that of flutamide. However, the lipophility of *p,p′*-DDE (log K_{ow} 6.51) is more than three orders of magnitude greater than flutamide (log K_{ow} 3.35) and vinclozolin (log K_{ow} 3.10). The strong relationship between this factor and the uptake constant (*64*), depuration rate constant (*65*), and bioconcentration factor in fish (*66*) probably

resulted in higher tissue concentrations of *p,p′*-DDE than vinclozolin or flutamide in our experiment. Determination of the relative potencies of these three chemicals will require investigation of chemical concentrations at the target tissues. This difference in lipophility may also explain the noteworthy effect pattern seen with the lowest dose of *p,p′*-DDE. Here, sexual behavior was almost eliminated, but no effects were seen in sperm count, GSI, or display coloration. It is reasonable to expect that *p,p′*-DDE is rapidly partitioned into tissues with a high lipid content, such as the brain, thus affecting behavior before other secondary sex characteristics. However, the response pattern of *p,p′*-DDE is also known to differ from those of vinclozolin and flutamide in the rat (*10,12,18*). Finally, it is possible that these differences are caused by subtle, tissue-dependent differences in AR affinities for antiandrogens as seen in other fish species (*24,25*).

Some of the measured parameters exhibited neutral or negative dose responses (i.e., the higher dose produced a weaker response than the low dose). From a traditional toxicologic viewpoint, this type of bell-shaped dose–response curve is somewhat surprising, although such responses are common in physiologic and in certain hormone studies. A survey of the literature from the last decade revealed nearly 100 titles reporting this type of response in hormone research. Of particular relevance to the present study, Wong et al. (*11*) demonstrated that the M2 metabolite of vinclozolin binds the AR, producing a ligand that can enter the nucleus. The presence of even small quantities of the natural androgen dihydroxytestosterone distorted this ligand, preventing the induction of DNA transcription, making M2 a functional antiandrogen. However, in the absence of dihydroxytestosterone, DNA transcription proceeded and M2 functioned as an androgen analogue, leading the authors to suggest that M2 functions as an androgen at high *in vivo* concentrations.

In conclusion, this study demonstrates that *p,p′*-DDE, vinclozolin, and flutamide caused profound demasculinization of fully matured male guppies, impairing male sexual characteristics from the cellular level to the organismal level after only 30 days of exposure. In a parallel study, Bayley et al. (*63*) measured the same end points in adult male guppies after exposure to the same three chemicals throughout the juvenile period. Similar results were obtained for sperm count and courtship behavior, but the actual size of the testis (GSI) was unaffected by the treatments. In addition, juvenile exposure caused delayed sexual maturation and a skewed sex ratio toward female predominance at adulthood in treated groups.

The impairments of the guppy's sexual characteristics are consistent with an antiandrogenic action of vinclozolin and *p,p′*-DDE. However, it is noteworthy that some of the measured end points, including body coloration and sexual behavior, responded similarly to estrogenic compounds (*31,35*), suggesting that demasculinizing and feminizing endocrine disruptors may have common molecular targets and/or cellular responses. Studies of the interactions between these chemicals and sex steroid receptors are required in the guppy to provide concrete evidence of the mechanisms underlying these effects on the sexual phenotype.

A number of fundamental questions remain unanswered. First, we need to confirm or disprove our assumption that the measured changes in the male sexual characteristics are actually translated into an impaired reproduction. Also, the possible effects of vinclozolin and *p,p′*-DDE on female fertility should be investigated, for instance, by mating exposed males with unexposed females and vice versa. Finally, long-term exposure to environmentally realistic concentrations, involving several generations and all life stages, should be carried out. These studies are currently being performed in our laboratory.

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Agricultural Pesticide Use in California: Pesticide Prioritization, Use Densities, and Population Distributions for a Childhood Cancer Study

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Several studies have suggested an association between childhood cancer and pesticide exposure. California leads the nation in agricultural pesticide use. A mandatory reporting system for all agricultural pesticide use in the state provides information on the active ingredient, amount used, and location. We calculated pesticide use density to quantify agricultural pesticide use in California block groups for a childhood cancer study. Pesticides with similar toxicologic properties (probable carcinogens, possible carcinogens, genotoxic compounds, and developmental or reproductive toxicants) were grouped together for this analysis. To prioritize pesticides, we weighted pesticide use by the carcinogenic and exposure potential of each compound. The top-ranking individual pesticides were propargite, methyl bromide, and trifluralin. We used a geographic information system to calculate pesticide use density in pounds per square mile of total land area for all United States census-block groups in the state. Most block groups (77%) averaged less than 1 pound per square mile of use for 1991–1994 for pesticides classified as probable human carcinogens. However, at the high end of use density (> 90th percentile), there were 493 block groups with more than 569 pounds per square mile. Approximately 170,000 children under 15 years of age were living in these block groups in 1990. The distribution of agricultural pesticide use and number of potentially exposed children suggests that pesticide use density would be of value for a study of childhood cancer. *Key words*: agriculture, childhood cancer, ecologic study, epidemiologic study, exposure assessment, geographic information systems, pesticides, risk assessment. *Environ Health Perspect* 109:1071–1078 (2001). [Online _____]
http://ehpnet1.niehs.nih.gov/docs/2001/109p1071-1078gunier/abstract.html

Some epidemiologic studies suggest an association between pesticide exposure and childhood cancer (*1,2*). Most studies have used questionnaires to evaluate parental occupational exposure around the time of the child's birth and exposure to the parents or child from pesticide use in the home and garden. Such information is potentially limited by response bias. Childhood cancer has not been evaluated with respect to potential exposure to agricultural pesticides because respondents are unlikely to have specific knowledge about pesticide use on nearby fields.

Few tools exist for identifying regions with a high density of agricultural pesticide use. County crop acreage totals are available, but land use varies tremendously within California counties because of differences in topography and urbanization. Some investigators have used satellite imagery and a geographic information system (GIS) to identify the location of agricultural fields (*3,4*). These indices provide information on the population living near fields, but only indirect estimates of pesticide use based on crop type. The resulting pesticide use estimates are limited by crop misclassification and the assumption that all fields are treated similarly for a given crop. Some studies of cancer in adults have been conducted with pesticide use data summarized at the county level (*5–7*). However, the number of residents living near agricultural fields and the amount of specific pesticides applied agriculturally in these communities have generally not been available.

In 1992, California accounted for 22% of all agricultural pesticide use in the United States (*8*). There has been some form of pesticide use reporting in California for several decades, although before 1990 reporting was limited to applications that were restricted and required a permit. The California legislature mandated the Pesticide Use Report (PUR) system in 1990 (*9*), legally requiring growers and applicators to report all commercial agricultural pesticide use. Every month, written or electronic records of all pesticide applications are submitted to the county agricultural commissioners. The California Department of Pesticide Regulation (Sacramento) collects the data entered by the counties and after checking for errors makes it available to the public annually for a small fee. Few states have a full pesticide use reporting system and no other state has been collecting data since 1990. An important feature of the PUR data is that they provide the pounds of active ingredient applied. There are more than 850 pesticide active ingredients applied agriculturally in California each year. Inert ingredients, which might also be toxic, are not reported. The active ingredients, which we refer to as pesticides, range from compounds with no known carcinogenic potential to substances known to cause cancer in laboratory animals (*10*).

The PUR data provide an opportunity to develop more geographically precise estimates of agricultural pesticide use, which may be evaluated in conjunction with cancer incidence rates. California is particularly suited for such an analysis because it also has a statewide cancer reporting system. We focused on potential exposures to children because the latency period for childhood cancer is shorter than for adult cancer. For this statewide analysis, we grouped pesticides into toxicologic categories and chemical classes to account for compounds that might act similarly in the human body or in the environment. In addition, we prioritized individual pesticides by weighting the reported pounds of use by the potential of the pesticide to cause cancer and the possibility of exposure based on volatilization and environmental persistence. The geographic boundaries for which agricultural pesticide use is reported in California do not match the census boundaries. We developed GIS methods to summarize agricultural pesticide use by census-block group and estimated the number of children living in the upper 10th percentile of pesticide use density. Although we focused on childhood cancer and potential carcinogens, these methods could be modified for other health outcomes and populations.

Methods

PUR data. We used the 1991–1994 PUR data to coincide with the time period of the census and cancer incidence data, and because it represents the first few years of full pesticide use reporting. The PUR database provides the active ingredient, quantity applied, acres treated, crop treated, and date and location

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We thank the Department of Pesticide Regulation for providing valuable comments, S. Seidel and A. Ujihara for reviewing drafts, and T. Saunders for preparation of the manuscript.

This study was funded by grant R01 CA71745 from the National Cancer Institute.

The opinions expressed are the views of the authors. They do not necessarily reflect the official position of the California Department of Health Services.

Received 12 January 2001; accepted 19 March 2001.

for all agricultural pesticide applications. The locations of pesticide applications are reported using an identifier that represents a section within the Public Land Survey System (PLSS). The PLSS is a nationwide survey that grids the land in each state into approximately 1-square-mile rectangular units called sections. Some areas of California were not surveyed when California became a state because of Spanish land grants. We used a version of the PLSS with the grid lines extended to cover any areas that were not surveyed (*11*). We checked for and deleted from further analysis applications with reported section identifiers that did not correspond to a valid section identifier within the PLSS.

A small percentage of data entry errors have been reported in the PUR that result in erroneously large amounts of pounds applied (*12*). We developed methods to identify and correct errors in the quantity of pesticide applied that could misclassify exposure. We used the application rate (pounds per acre) to identify potential reporting errors with unreasonably high quantities of pesticide applied. We calculated the mean application rate for each pesticide using the 1995 PUR data. We used the 1995 data for quality control because this was the most recent year available and had the fewest number of extremely high application rates. We assigned each pesticide an estimated maximum allowable application rate that was at least 2 standard deviations above its 1995 mean rate. Application rates above the estimated maximum allowable are generally so large as to be economically unfeasible. An application in the PUR database was considered an error if the application rate was greater than the maximum allowable rate for that pesticide. We checked these errors in two counties and found that they were largely the result of entry errors or illegible reporting from the growers (*13*). We recalculated the quantity of pesticide applied for these potentially erroneous applications by multiplying the acres treated by the maximum allowable rate.

Pesticide use by groups. We combined pesticides from the PUR data into four toxicologic groups for our statewide analysis: probable carcinogens, possible carcinogens, genotoxic compounds, and reproductive or developmental toxicants. We identified 73 pesticides for these four groups from all active ingredients reported to the PUR statewide from 1991 to 1994 (Table 1). Some individual pesticides were included in more than one group. The U.S. Environmental Protection Agency (EPA) classifies most pesticides according to their human carcinogenic potential (*10*). California banned or severely restricted the agricultural use of all pesticides classified as known human carcinogens (class A) or probable human carcinogens with limited human evidence (class B1) before 1991. For the purposes of this study, we created one toxicologic group with 19 pesticides classified as probable human carcinogens with sufficient evidence in laboratory animals (class B2). We formed a second group with 37 compounds categorized as possible human carcinogens with limited evidence in animals (class C).

Some pesticides are not classified as carcinogens but have evidence of other types of toxicity that may be relevant. Genotoxic chemicals have demonstrated the ability to directly damage DNA. Several laboratory tests are commonly used to assess genetic toxicity, including gene mutation, chromosome aberration, sister chromatid exchange, and DNA damage. We chose 27 pesticides with at least two positive results in genetic toxicity assays for a third toxicologic group (*14,15*). Because many childhood cancer cases occur shortly after birth and may be related to perinatal exposures, reproductive and developmental toxicants were also of interest. We selected 19 pesticides with at least one positive result in reproductive or developmental studies conducted in laboratory animals to form a final group for analysis (*16*).

We combined pesticides into four additional groups based on chemical class (organochlorides, organophosphates, carbamates, and dithiocarbamates). We identified chemical classes using a pesticide dictionary and chemical structure (*17*). There were 36 pesticides with reported use between 1991 and 1994 in these four classes. Organochloride insecticides had mostly been replaced by organophosphates by 1990, so these represent the smallest and largest groups, respectively. Table 1 provides a list of pesticides in each chemical class.

Pesticide cancer hazard prioritization. Although some low-use pesticides may be highly toxic, for an epidemiologic study a minimum amount of use is required to provide enough power to detect a risk. Therefore, we determined a minimum annual average use based on the land area of California, which is approximately 150,000 square miles. We considered average statewide use greater than one pound per square mile to be significant, and chose 150,000 pounds as a minimum annual average statewide use for consideration in this analysis. Thirty-eight pesticides from the toxicologic groups met this minimum annual use.

Table 1. Pesticides with reported use in California, 1991–1994, in toxicologic and chemical groups.

Toxicologic and chemical groups	Pesticides
Probable carcinogens (class B2)[a]	Alachlor, cacodylic acid, captan, chlordane, chlorothalonil, daminozide, 1,3-dichloropropene, iprodione, lindane, mancozeb, maneb, metam sodium, orthophenylphenol, oxythioquinox, propargite, propoxur, pentachlorophenol, propyzamide, vinclozolin
Possible carcinogens (class C)[b]	Acephate, acrolein, amitraz, atrazine, benomyl, bifenthrin, bromacil, bromoxynil, carbaryl, chlorthal-dimethyl, cyanazine, cypermethrin, dichlobenil, dichlorvos, dicloflop-methyl, dicofol, dimethoate, ethalfluralin, fosetyl-al, hydrogen cyanamide, imazalil, linuron, methidathion, metolachlor, molinate, norflurazon, oryzalin, oxadiazon, oxyfluorfen, pendimethalin, permethrin, phosmet, phosphamidon, piperonyl butoxide, simazine, triadimefon, trifluralin
Genotoxic compounds[c]	2,4-Diethylamine, acephate, alachlor, aldicarb, atrazine, benomyl, captan, carbaryl, carbofuran, chlordane, chloropicrin, chlorothalonil, chlorpyrifos, diazinon, 1,3-dichloropropene, diquat dibromide, malathion, metam sodium, methyl bromide, methyl parathion, mevinphos, orthophenylphenol, oxydemeton, methyl, paraquat dichloride, pentachlorophenol, trifluralin, ziram
Developmental or reproductive toxicants[d]	2,4-Diethylamine, benomyl, bromoxynil, carbofuran, cyanazine, diazinon, diquat dibromide, *s*-ethyl dipropylthiocarbamate (EPTC), mancozeb, maneb, metam sodium, methyl bromide, methyl parathion, oxyfluorfen, propargite, *s,s,s*-tributyl, triadimefon, vinclozolin
Organochlorides[e]	Dicofol, endosulfan, lindane
Organophosphates[e]	Acephate, azinphos-methyl, chlorpyrifos, diazinon, dimethoate, disulfoton, ethoprop, fonofos, malathion, methamidophos, methidathion, methyl parathion, mevinphos, naled, oxydemeton-methyl, parathion, phorate, phosmet, profenofos
Carbamates[e]	Aldicarb, benomyl, carbaryl, carbofuran, frometanate, methomyl, pebulate, propoxur
Dithiocarbamates[e]	Mancozeb, maneb, metam sodium, thiram, zineb, ziram

[a]Probable human carcinogens with sufficient evidence in laboratory animals and inadequate or no evidence in humans (*10*). [b]Possible human carcinogens with limited evidence in laboratory animals (*10*). [c]Positive in two or more laboratory assays (*14,15*). [d]Positive in one or more developmental or reproductive studies in laboratory animals (*16*). [e]Chemical groups were identified from Meister (*17*).

To prioritize individual pesticides for analysis, we developed a hazard weighting system based on two measures of carcinogenic potential and two measures of exposure potential. We assigned weights for each of these attributes to the highest-use pesticides from the toxicologic groups. The U.S. EPA cancer class was used to assign to each pesticide a weight from 1 through 10 based on the evidence that it is a carcinogen (*10*). Since there were no class A or class B1 carcinogens with geographically referenced use during our study period, the highest score assigned for cancer class was 7. Cancer slope factors, which estimate cancer potency from the dose–response relationship, have been calculated for all probable (class B2) and most possible (class C) carcinogens (*10*). As a second measure of carcinogenic potential, we assigned each pesticide a weight from 1 to 10 based on its cancer potency. If data were not available, a default weight of 1 was assigned to the pesticide for that attribute. Table 2 provides a key to the weights for each attribute.

We used volatilization flux rate and field half-life as measures of physical characteristics that could be associated with exposure potential. Volatilization flux estimates the tendency of a pesticide to move into the air after application and is correlated with the downwind concentration in air (*18*). We estimated the volatilization flux for each pesticide using the vapor pressure, water solubility, and soil absorption coefficient (*19,20*). Pesticides were assigned a weight from 1 through 10 based on the calculated volatilization flux. We used the field dissipation half-life—a measure of the overall rate of disappearance of a pesticide from treated fields—as an indicator of persistence (*20*). Pesticides were assigned a weight from 1 through 5 based on persistence. The range used for persistence weight was half that used for volatilization flux weight because the dose received by children from ingestion of household dust is estimated to be about half the dose from inhalation for most pesticides (*21,22*). Moreover, we considered volatilization and secondary drift a necessary precursor for most potential exposures to children in nearby communities.

We calculated the cancer hazard factor for each pesticide by multiplying the weights for each attribute and then normalizing to make the highest possible score 10. The range of potential cancer hazard factors covers almost four orders of magnitude (0.002 to 10):

$$\text{Cancer Hazard Factor} = (\text{Class} \times \text{Potency} \times \text{Flux} \times \text{Persistence})/500.$$

We calculated hazard-adjusted pesticide use by multiplying the pounds applied by the corresponding cancer hazard factor. Individual pesticides were ranked by hazard adjusted use:

$$\text{Hazard Adjusted Pesticide Use} = \text{Cancer Hazard Factor} \times \text{Pounds of Use}.$$

Block-group exposure assessment. We used the 1991–1994 PUR data to calculate the annual average pesticide use in pounds for each square-mile section (*23*). We used the annual average because our focus was on cancer and chronic exposure. We used a GIS to determine the spatial relationship between sections and census-block groups. In 1990, California block groups had a median land area of 0.2 square miles and a huge range, from 0.001 to 3,610 square miles (*24*). Pesticide use was allocated from the section to each corresponding block group on the basis of percent area of the section in that block group. We calculated pesticide use density in pounds per square mile of census-block group by summing the average pounds applied in all relevant sections and then dividing by the block-group area. The median, 90th percentile, and maximum block-group pesticide use density were determined for each pesticide and pesticide group. We used 1990 census data to obtain the number of children under 15 years of age by block group. The number of children living in block groups with pesticide use density above the 90th percentile was calculated for each pesticide group and the highest cancer hazard ranking pesticides.

Results

PUR data. For all pesticides reported in the PUR, the annual average agricultural pesticide use for 1991–1994 was greater than 169 million pounds. Correcting for application rates above the estimated maximum allowable rate reduced the average by 5% to 160 million pounds. Application rate errors were often an order of magnitude greater than the average rate, indicating data entry errors. Location errors further reduced statewide annual average pesticide use by another million pounds or less than 1%. The most frequent location error involved sections that were not within the reported county. Location errors occurred in more than 1,000 sections (0.5%) and affected a smaller number of pounds than high application rate errors. Given the size of the PUR database, we considered the observed error rate of approximately 6% of reported pounds relatively low.

Pesticide use by groups. The statewide average annual use for the pesticide groups is shown in Table 3. The probable and possible carcinogen groups each had about 10 million pounds per year of reported use, and the genotoxic and developmental/reproductive toxicant groups were both greater than 30 million pounds per year. Among the chemical classes, organochloride insecticides had the least use with less than 1 million pounds per year, and the dithiocarbamate fungicides had the most use with greater than 10 million pounds per year.

To evaluate changes in pesticide use from 1991 to 1994, we graphed annual reported use for probable carcinogens, possible carcinogens, methyl bromide, and metam sodium (Figure 1). We chose methyl bromide and metam sodium because these were the highest use pesticides from the four toxicologic groups. The use of probable carcinogens (class B) increased from 8 to 16 million pounds from 1991 to 1994. Most of that increase was caused by metam sodium use, which grew from approximately 5 million to 11 million pounds. The largest increase occurred between 1991 and 1992,

Table 2. Pesticide cancer hazard weights by attribute.

Weight	Cancer class[a]	Cancer potency[a] (mg/kg/day)	Volatilization flux[b,c]	Field half-life[c] (days)
10	A	> 1	$> 10^{-1}$	—
8	B1	> 0.1–1	$>10^{-3}$–10^{-1}	—
7	B2	—	—	—
5	C	> 0.01–0.1	$>10^{-5}$–10^{-3}	> 100
4	—	—	—	76–100
3	G or D/R[d]	0.001–0.01	10^{-7}–10^{-5}	51–75
2	—	—	—	26–50
1	NA	< 0.001 or NA	$< 10^{-7}$ or NA	< 25 or NA

NA, not available.
[a]From U.S. EPA (*10*). [b]Flux rate = vapor pressure/(water solubility × soil absorption coefficient) from Glotfelty et al. (*19*). [c]Vapor pressure, water solubility, soil absorption, and field half-life from U.S. Department of Agriculture (*20*). [d]Genotoxic or developmental/reproductive toxicant (*16*).

Table 3. Average annual pesticide use in California from 1991 to 1994 for pesticide groups.[a]

Pesticide group	Average pounds[b]
Class B carcinogens	12,643,173
Class C carcinogens	9,972,335
Genotoxic compounds	36,445,168
Developmental/reproductive toxicants	31,472,459
Organochlorides	903,550
Organophosphates	6,687,806
Carbamates	2,326,545
Dithiocarbamates	10,884,652

[a]Individual pesticides can be in more than one group. [b]PUR data corrected for erroneously high application rates and includes only valid geographic locations.

which may reflect increased awareness of the legal mandate of reporting (*12*). This time period also coincides with severe restrictions on the use of 1,3-dichloropropene (Telone), a fumigant that was largely replaced by metam sodium. The use of possible carcinogens (class C) and methyl bromide remained relatively constant.

Pesticide cancer hazard prioritization. The calculated cancer hazard factors for individual pesticides (Table 4) ranged over more than two orders of magnitude, although most pesticides had hazard factors between 0.1 and 1.0. For pesticides classified as probable or possible carcinogens, the cancer hazard weights are greater than the exposure potential weights because of the lesser weighting for persistence. The cancer hazard factors for pesticides from the other toxicologic groups were more influenced by their exposure potential.

The relative ranking of pesticide use changed significantly when pounds were adjusted by the cancer hazard factors. The top pesticides in the state ranked by hazard-adjusted use (Table 5) were propargite, methyl bromide, and trifluralin. The top pesticides from the toxicologic groups ranked by pounds alone were methyl bromide, metam sodium, and chlorpyrifos. Propargite had a larger cancer hazard factor than some high-use pesticides, such as chlorpyrifos, producing a much higher ranking by hazard-adjusted use.

Block-group exposure assessment. We calculated the statewide distribution of pesticide use density among block groups with more than 1 pound per square mile of use for a given pesticide group or individual pesticide (Table 6). Very low pesticide use densities may have been the result of location errors within counties that could not be eliminated. Therefore, we considered block groups with use densities less than 1 pound per square mile to have little potential exposure. There were 3,000–9,000 census-block groups in the state with more than 1 pound per square mile of pesticide use for each pesticide group. The median densities were generally greater than 10 pounds per square mile. The distributions were not normal with order-of-magnitude increases between the median, 90th percentile, and maximum use densities. The 90th percentile of use density was around 500 pounds per square mile for the two carcinogen groups and greater than 1,500 pounds per square mile for the genotoxic and developmental or reproductive toxicant groups. Among the chemical classes, organochlorides had the lowest use density and dithiocarbamates had the highest, with a median of 30 pounds per square mile.

For individual pesticides, the number of block groups with more than 1 pound per

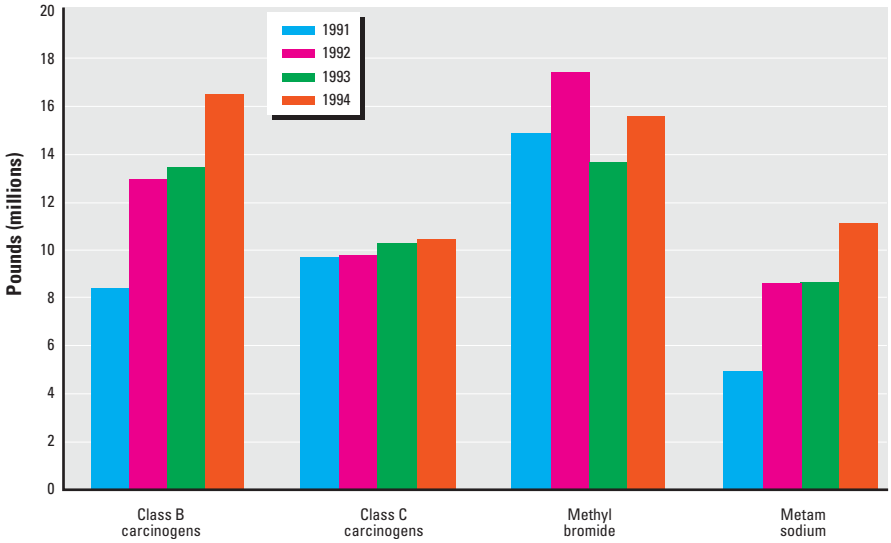

Figure 1. Annual agricultural pesticide use in California from 1991 to 1994. Metam sodium is included among Class B carcinogens. PUR data corrected for erroneously high application rates and includes only valid geographic locations.

Table 4. Cancer hazard weights and factors for pesticides in toxicologic groups with annual use > 150,000 pounds per year.

Pesticide	Cancer class weight	Cancer potency weight	Volatilization flux weight	Field half-life weight	Cancer hazard factor[a]
Probable carcinogens					
Captan	7	3	5	1	0.210
Chlorothalonil	7	3	8	2	0.672
Iprodione	7	5	5	1	0.350
Mancozeb	7	5	1	2	0.140
Maneb	7	5	1	2	0.140
Metam sodium	7	8	1	1	0.112
Propargite	7	5	5	4	1.400
Possible carcinogens					
Acephate	5	5	3	1	0.150
Carbaryl	5	5	5	1	0.250
Chlorthal-dimethyl	5	3	8	3	0.720
Cyanazine	5	10	3	2	0.600
Dicofol	5	8	5	3	1.200
Dimethoate	5	1[b]	5	1	0.050
Fosetyl-al	5	1[b]	1	1	0.010
Methidathion	5	1[b]	8	1	0.080
Metolachlor	5	1[b]	8	5	0.400
Molinate	5	8	10	1	0.800
Norflurazon	5	1[b]	5	5	0.250
Oryzalin	5	8	5	1	0.400
Oxyfluorfen	5	8	5	2	0.800
Pendimethalin	5	1[b]	8	5	0.400
Permethrin	5	5	5	2	0.500
Phosmet	5	1[b]	5	1	0.050
Simazine	5	8	5	4	1.600
Trifluralin	5	3	10	4	1.200
Genotoxic or developmental/ reproductive toxicants					
2,4-Diethylamine	3	1[b]	5	2	0.060
Aldicarb	3	1[b]	5	2	0.060
Carbofuran	3	1[b]	5	2	0.060
Chloropicrin	3	1[b]	10	1	0.060
Chlorpyrifos	3	1[b]	8	2	0.096
Diazinon	3	1[b]	5	1	0.030
Ethyl dipropylthiocarbamate	3	1[b]	10	1	0.060
Malathion	3	1[b]	5	1	0.030
Methyl bromide	3	1[b]	10	2	0.120
Mevinphos	3	1[b]	5	1	0.030
Paraquat dichloride	3	1[b]	1	5	0.030
S,S,S-tributyl	3	1[b]	5	2	0.060
Ziram	3	1[b]	5	2	0.060

[a]Cancer hazard factor = (evidence weight × potency weight × flux weight × persistence weight)/500. [b]Not available.

square mile of use varied tremendously from 194 for molinate to > 3,400 for methyl bromide. The 90th percentile of use density was greater than 100 pounds per square mile for most individual pesticides. The soil fumigants methyl bromide and metam sodium had much higher use densities than the other individual pesticides with 90th percentile values greater than 1,500 pounds per square mile.

To illustrate the methods used to calculate block-group pesticide use density, an example is provided from Fresno, California. Figure 2A shows probable carcinogenic pesticide use in pounds by section and Figure 2B shows the resulting use density in pounds per square mile for census-block groups in this area. The block-group pesticide use density essentially follows the section-level pesticide use. Figure 2B also illustrates that larger, rural block groups tend to have the highest pesticide use density and smaller, urban block groups the lowest. In high-use rural areas, census-block–group mapping is less geographically specific than mapping by section because of the large area of these block groups.

We mapped the geographic distribution of pesticide use density by block group using the percentiles of the statewide distribution for all probable carcinogens (Figure 3) and for propargite, which was the highest-ranking individual compound (Figure 4). For probable carcinogens, the highest use areas were in the San Joaquin, Sacramento, Salinas, and Imperial Valleys. This corresponds well with the heaviest agricultural counties in the state based on farm revenues (*25*). Propargite use was not as geographically widespread, and the high-use density area was primarily the San Joaquin Valley.

More than 6.6 million children under 15 years of age lived in California in 1990. The number of children living in block groups above the 90th percentile of use density varied considerably among the pesticide groups and individual pesticides (Table 6). Developmental or reproductive toxicants had the most children with nearly 267,000, and molinate had the least number of children with just over 3,300. Organophosphates and organochlorides had about 200,000 and 60,000 children living in these high-use block groups, respectively. The variation in the number of children living in these block groups demonstrates that different populations were potentially exposed for each group and individual pesticide.

Table 5. Highest-ranking pesticides based on hazard-adjusted use, 1991–1994.

Pesticide	Cancer hazard factor	Corrected pounds[a]	Hazard-adjusted use[b]
Propargite	1.400	1,600,982	2,241,375
Methyl bromide	0.120	16,901,451	2,028,174
Trifluralin	1.200	1,230,218	1,476,262
Simazine	1.600	869,962	1,391,939
Molinate	0.800	1,380,424	1,104,339
Metam sodium	0.112	8,300,569	929,664
Dicofol	1.200	554,077	664,892
Chlorothalonil	0.672	786,572	528,576
Chlorthal-dimethyl	0.720	642,891	462,882
Oxyfluorfen	0.800	334,325	267,460
Oryzalin	0.400	667,445	266,978
Cyanazine	0.600	411,331	246,799
Chlorpyrifos	0.096	2,429,610	233,243
Carbaryl	0.250	820,487	205,122
Iprodione	0.350	408,562	142,997
Chloropicrin	0.060	2,364,831	141,890
Pendimethalin	0.400	284,845	113,938
Permethrin	0.500	201,795	100,898
Ziram	0.060	1,590,812	95,449
Captan	0.210	417,612	87,699

[a]PUR data corrected for erroneously high application rates. [b]Hazard adjusted use = corrected pounds × cancer hazard factor.

Table 6. Distribution of annual average agricultural pesticide-use density in California census-block groups for toxicologic groups, chemical groups, and high-hazard individual pesticides.[a]

	Block groups[b]	Median (lbs/mi^2)	90th percentile (lbs/mi^2)	Max (lbs/mi^2)	Children (< 15 years) in 90th percentile[c]
Toxicologic groups					
Class B	4,932	31	569	14,935	169,884
Class C	6,218	23	445	5,043	198,375
Genotoxic	7,505	48	1,844	70,670	261,333
Developmental/reproductive	6,647	45	1,789	48,784	266,960
Chemical groups					
Organochlorides	3,881	9	86	589	60,909
Organophosphates	9,268	18	349	7,129	204,144
Carbamates	6,755	14	141	1,706	139,316
Dithiocarbamates	3,216	30	764	14,931	109,474
Individual pesticides					
Propargite	2,144	21	172	926	61,892
Methyl bromide	3,431	163	2,668	45,185	127,562
Trifluralin	1,287	14	118	784	35,983
Simazine	2,109	15	112	582	64,462
Molinate	194	49	696	1,433	3,334
Metam sodium	1,072	86	1,503	14,480	42,145
Dicofol	1,342	7	72	352	44,902
Chlorothalonil	**2,359**	**13**	**109**	**2,537**	**84,740**

[a]Calculated from census-block groups with use density > 1 lb/mi^2 for that pesticide. [b]Number of block groups with > 1 lb/mi^2 use density for that pesticide; total block groups used in this analysis were 21,443. [c]Number of children under 15 years of age living in census-block groups above the 90th percentile of pesticide-use density.

Discussion

We developed methods to quantify agricultural pesticide use density for census-block groups using the PUR data and a GIS. In California, there was a wide range of pesticide use density (Table 6). Most block groups in the state (57–99%) averaged less than 1 pound per square mile of average annual use (1991–1994) for pesticide groups and individual pesticides. However, at the high end of the distribution (> 90th percentile), pesticide use density often exceeded 1,000 pounds per square mile. More than 100,000 children lived in these high-use density block groups for most pesticide groups and about 50,000 children for individual pesticides.

The interrelationship of agricultural pesticide use, individual environmental exposure, and health effects has not been well defined. The limited environmental and biologic monitoring data available suggest that residents may be exposed to pesticides applied agriculturally through multiple routes. Researchers have detected pesticides in ambient air near agricultural fields in California and throughout the United States (*26–28*). Dermal contact and ingestion of household dust are important exposure routes for young children (*29–33*). Well monitoring has also identified pesticides in the groundwater of agricultural communities in the state (*34*). Biologic monitoring of pesticide levels in children indicated an inverse relationship with distance from treated orchards (*35,36*).

These findings suggest that the hundreds of thousands of children living in areas with high agricultural pesticide use have a greater potential for exposure than their more urban

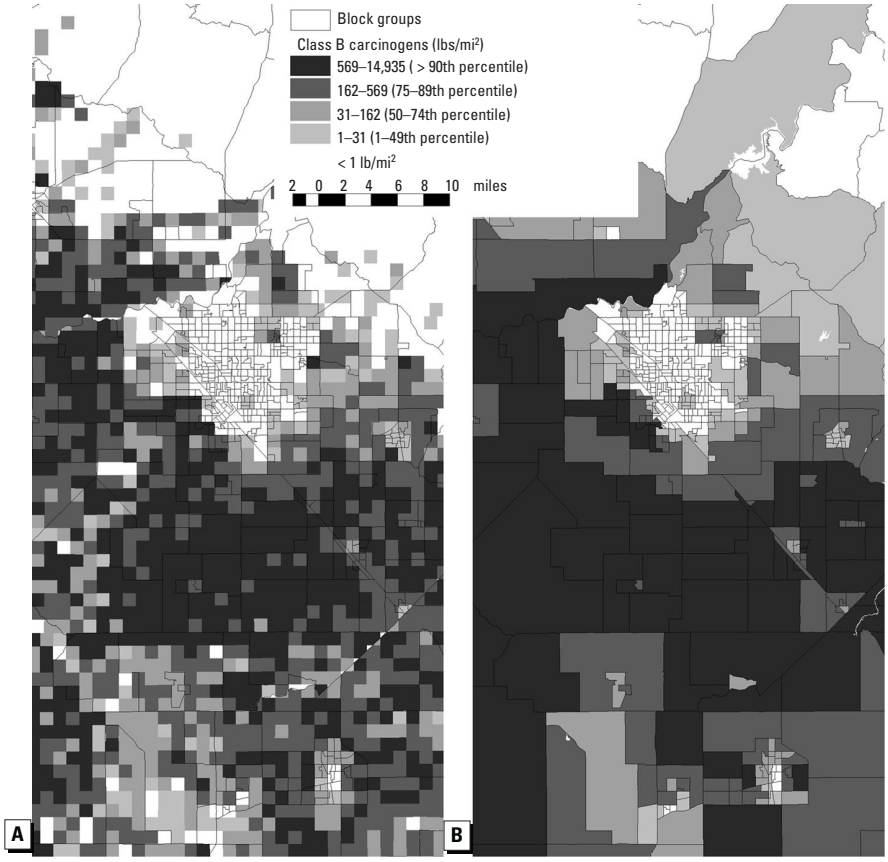

Figure 2. Annual average class B pesticide use, in pounds per square mile, around Fresno, California, as reported to (*A*) a section of the public land survey system, and (*B*) a census-block group, 1991–1994.

counterparts. Population growth in California has led to the development of suburban areas adjacent to fields or on former farmland, increasing the potentially exposed population. We consider pesticide use density an indicator for a wide range of potential exposure pathways, including inhalation of ambient air, soil drift and persistence in household dust, potential groundwater contamination, parental occupational "take home" exposures, playing in fields, and eating produce directly from treated fields.

Hazard-weighted pesticide use created different priorities for assessing individual compounds (Table 5). Our focus was on ranking carcinogens for a childhood cancer study, but these hazard-weighting methods could be modified for other health outcomes of interest (*37,38*). A hazard scoring system used by the Department of Pesticide Regulation to evaluate pesticides as toxic air contaminants also ranked propargite, simazine, chlorothalonil, molinate, metam sodium, cyanazine, and chlorpyrifos among the top 20 compounds (*39*). Methyl bromide, trifluralin, carbaryl, and captan are already classified as toxic air contaminants in California pursuant to section 14021(b) of the Food and Agricultural Code (*39*). Nonoccupational exposures to molinate are suggested to exceed safety margins (*40*). Methyl bromide, chlorothalonil, and molinate have been detected in ambient air of agricultural communities in California (*27*).

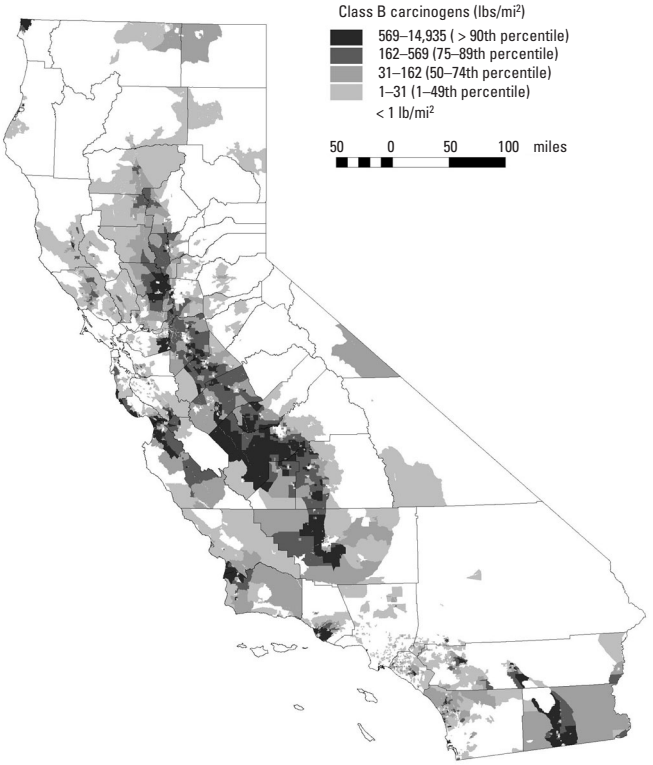

Figure 3. Annual average class B pesticide use density in California census-block groups, 1991–1994, in pounds per square mile.

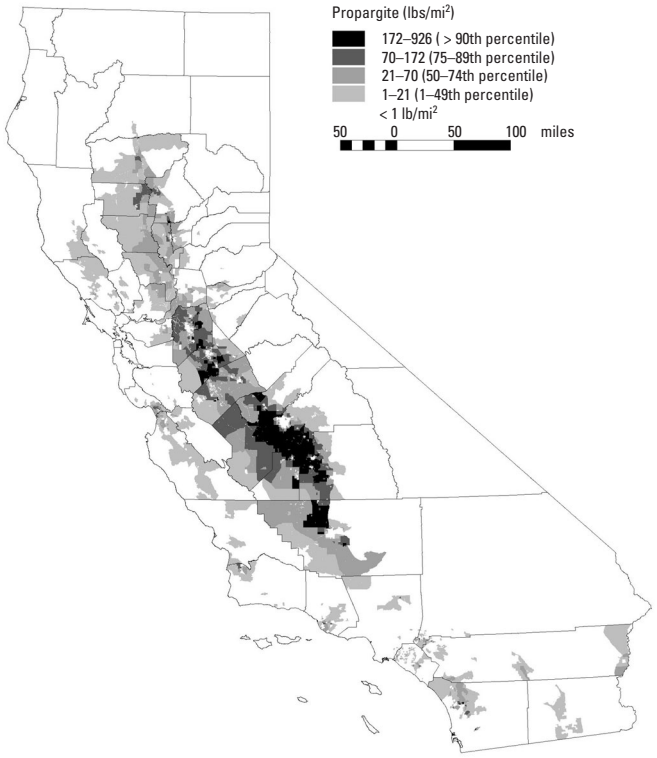

Figure 4. Annual average propargite use density in California census-block groups, 1991–1994, in pounds per square mile.

There are some notable limitations to weighting pesticide use by cancer hazard. Pesticides that have not been toxicologically tested for carcinogenicity, genotoxicity, or developmental/reproductive toxicity were not included in our prioritization. Eleven pesticides with more than 1 million pounds per year of use in California had insufficient toxicologic and environmental data for hazard weighting (sulfur, petroleum oil, sodium chlorate, copper hydroxide, mineral oil, copper sulfate, chloropicrin, petroleum distillates, sulfuryl fluoride, calcium hydroxide, and diuron). Furthermore, the weighting of each hazard attribute and exposure relative to carcinogenicity may not reflect true environmental and biologic activity. Animal cancer potency may not accurately reflect the potency for humans, although the evidence is fairly convincing that human carcinogens are carcinogenic in rodents (*41*). Some pesticides degrade into compounds that have more or less carcinogenic or exposure potential than the original parent compound. For example, the actual fumigant action of metam sodium (a probable carcinogen) comes from a reaction product called methyl isothiocyanate, which is not thought to be a carcinogen. Information on the environmental breakdown products of pesticide active ingredients was not included in our prioritization system because data were not available for most pesticides.

The PUR system has some limitations that are potentially problematic for epidemiologic studies. Information on residential pesticide use in the home and garden is not collected. Agricultural pesticide use is reported to a square-mile section, but air monitoring data from application sites suggest that pesticide concentrations may decrease significantly within a mile (*18,42–44*). Nonagricultural pesticide applications, including structural fumigations and landscaping uses on golf courses and along highways, are reported only at the county level in the PUR data. Improved spatial resolution for both agricultural and structural/landscaping applications would represent a significant refinement to the PUR system for use in health studies. The PUR system is legally mandated, but pesticide use is self-reported, and underreporting has not been evaluated. Information on the type and amount of inert ingredients applied is not provided. Many of the solvents used in pesticide formulations also have toxicologic effects of concern (*45,46*). Despite these limitations, the PUR system is still probably the most comprehensive agricultural pesticide use database in the world (*12*).

We calculated the annual average pesticide use density to examine chronic exposure. However, pesticide applications are frequently seasonal, and many are applied only once per year or in response to specific pest infestations. If the PUR data are to be used for studies of other health outcomes, the relevant time period should be considered. Because of the geographic resolution of the PUR data, we assumed that pesticide use was distributed evenly within a square-mile section. Pesticide use density represents pesticide use averaged over the entire land area of the block group, but all applications could have occurred in a single section.

The PUR data represent an extremely valuable resource for conducting health studies. Residents are unlikely to have knowledge about pesticide use on nearby fields, unlike home and garden use. The measures presented here are based on independent reporting and do not rely on recall by study participants. The PUR data also allowed for evaluation of specific pesticide active ingredients and the combination of pesticides with similar chemical or toxicologic properties. For other health studies, pesticide groups should be tailored to the health outcomes or exposure pathways of interest. A GIS was essential in conducting this analysis because it allowed for the spatial overlay of agricultural pesticide use and census-block groups.

The heavy use of potentially toxic agricultural pesticides in some areas of California warrants further exposure and epidemiologic investigation. Environmental and biologic monitoring is needed to determine the relationship between agricultural pesticide use and individual exposure. Additional toxicologic data are also desirable for many high-use pesticides. The range of values reported here for census-block group pesticide use density are suitable for a statewide epidemiologic study of childhood cancer. The number of children living in both high and low pesticide-use density areas is sufficient to allow for statistical testing between these groups (*47*). The pesticide-use density methods presented here can be used, with some minor modifications, in other health studies conducted at the block-group level in California or in other states if pesticide use reporting systems are developed.

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Iron Deficiency Associated with Higher Blood Lead in Children Living in Contaminated Environments

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The evidence that iron deficiency increases lead child exposure is based primarily on animal data and limited human studies, and some of this evidence is contradictory. No studies of iron status and blood lead levels in children have accounted for environmental lead contamination and, therefore, the source of their exposure. Thus, no studies have directly determined whether iron deficiency modifies the relationship of environmental lead and blood lead. In this study, we compared blood lead levels of iron-deficient and iron-replete children living in low, medium, or highly contaminated environments. Measurements of lead in paint, soil, dust, and blood, age of housing, and iron status were collected from 319 children ages 1–5. We developed two lead exposure factors to summarize the correlated exposure variables: Factor 1 summarized all environmental measures, and Factor 2 was weighted for lead loading of house dust. The geometric mean blood lead level was 4.9 μg/dL; 14% exceeded 10 μg/dL. Many of the children were iron deficient (24% with ferritin < 12 ng/dL). Seventeen percent of soil leads exceeded 500 μg/g, and 23% and 63% of interior and exterior paint samples exceeded 5,000 μg/g. The unadjusted geometric mean blood lead level for iron-deficient children was higher by 1 μg/dL; this difference was greater (1.8 μg/dL) after excluding Asians. Blood lead levels were higher for iron-deficient children for each tertile of exposure as estimated by Factors 1 and 2 for non-Asian children. Elevated blood lead among iron-deficient children persisted after adjusting for potential confounders by multivariate regression; the largest difference in blood lead levels between iron-deficient and -replete children, approximately 3 μg/dL, was among those living in the most contaminated environments. Asian children had a paradoxical association of sufficient iron status and higher blood lead level, which warrants further investigation. Improving iron status, along with reducing exposures, may help reduce blood lead levels among most children, especially those living in the most contaminated environments. *Key words*: **children, environmental exposure, epidemiology, iron deficiency, lead poisoning.** *Environ Health Perspect* **109:1079–1084 (2001). [Online _____]**
http://ehpnet1.niehs.nih.gov/docs/2001/109p1079-1084bradman/abstract.html

Childhood lead exposure is one of the most significant environmental health threats that affect children(*1–3*). Adverse effects of lead include cognitive deficits, neurotoxicity, behavior disorders, slowed growth, reduced heme synthesis, and impaired hearing (*1,3–9*). Although health and regulatory programs designed to reduce lead exposure are proving successful (*10*), many young children in the United States still have blood lead levels > 10 μg/dL, the Centers for Disease Control and Prevention (CDC) level of concern (*1,10–12*). The prevalence of elevated blood lead levels among minority, low-income inner-city children remains several times the national average (*10–12*). These same children are also more likely than others to be iron deficient, a condition that affects up to 6% of young children nationally (*13–16*), with insufficient iron intake in up to one-third of children in some communities (*17*).

It is biologically plausible that iron deficiency could lead to higher lead levels in children. Controlled animal studies consistently demonstrate higher lead levels in iron-deficient animals than in iron-replete controls (*18–23*). The mechanism for enhanced absorption is likely to be substitution of Fe^{+2} with Pb^{+2} and increased active transport into the body (*19,22,24,25*). Similarly, it is possible that Pb^{+2} may occupy vacant Fe^{+2} sites in the hematopoeitic system, thereby reducing lead excretion. Clinical studies of chelation therapy suggest that iron-deficient children may retain more lead in their bodies (*26,27*). It is also possible that iron deficiency modifies behavior, increasing pica or hand-to-mouth behavior in children and thereby increasing ingestion exposures to lead in their environment (*28,29*).

Despite the consistency of results in animal studies, the findings in human studies are less definitive. Experimental studies of iron deficiency and lead uptake in human adults are not consistent (*19,30–33*). Several epidemiologic studies in children support a correlation between iron deficiency and higher blood lead (*15,34–36*). Other studies have found no relationship between iron intake or low iron stores and blood lead in children (*37,38*); however, these studies either used diet to measure iron status (*38*) or studied older children (10–18 years) and did not control for age (*37*), which is an important factor affecting lead absorption (*39*).

To date, no studies examining iron status and blood lead in children account for environmental lead contamination, and thus the source of a child's exposure. Iron deficiency may be directly associated with lead uptake and systemic retention, or lead and iron deficiency may be independent factors, both of which may be related to another factor, such as poverty. Because the sociodemographic characteristics of children who are likely to be iron deficient also puts them at higher risk of lead exposure (*10*), it is not certain to what extent iron deficiency directly affects blood lead levels. Nor have any studies attempted to quantify the level of protection that sufficient iron status may confer on a child. In this study we evaluate whether iron deficiency is related to increased blood lead in children living in contaminated environments; we also account for major covariates, including socioeconomic status and child age.

Methods

Selection of households and participants. Participants in the study were part of an epidemiologic study of childhood lead exposure in Sacramento, California, one of three

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We thank M. Haan for collaboration in conduct of the survey, L. Zahler for superb coordination of fieldwork, R. McLaughlin for data management, J. Irias for blood lead and iron parameter measurements, P. Flessel and G. Guirguis for environmental laboratory measurements and quality control, and S. Samuels for statistical consultation. We appreciate the advice of R.D. Schlag, D.F. Smith, and R.R. Neutra, who reviewed the questionnaire and study design; S. Cummins and B. Abrams for reviewing drafts of this manuscript; and B. Lubin for assistance on iron deficiency parameters.

This research was supported in part by the California Department of Health Services Childhood Lead Poisoning Prevention Program; NIEHS award P01 ES09605 and EPA award R826709.

Received 3 July 2000; accepted 4 April 2001.

California sites studied by the California Department of Health Services (CDHS) from 1988 to 1990. We used information from the 1980 census to identify specific census tracts with many children between ages 1 and 6 years and a high prevalence of lead risk factors, including a high proportion of older housing, low income, and minority ethnicity. We selected specific census tracts after discussions with local health officials and firsthand observation. Eligible households were enumerated by door-to-door survey. Any household with a child between 1 and 6 years of age was considered eligible. Seventy-nine percent of 2,220 households in the study area were enumerated; 483 were eligible, and 232 households participated with a total of 382 children. Of the 382 children, 28 were missing information on environmental exposure and 35 were missing measurements of ferritin, a measure of iron status, for a total of 319 children for this analysis.

Environmental measurements of lead contamination. We collected up to three interior and three exterior paint samples from different areas of peeling and/or chipping paint. We collected paint samples from intact surfaces if there was no peeling or chipping paint available. Interior and exterior trim and porches were sampled in preference to walls and siding. We used the maximum interior and exterior paint lead level to characterize the dwelling. We collected front, side, and rear-yard soil samples from the top 2.5 cm or less of soil, and used the geometric mean of these soil lead levels for the data analysis. We collected house dust samples with a vacuum cleaner with an in-line filter trapping particles > 0.3 mm at 98% efficiency. Each sample was collected from the center of a room, with preference given to areas where children were reported to spend time. Values for both concentration of lead in house dust (micrograms per gram) and loading (amount of lead per unit area, micrograms per square meter) were reported. Environmental samples were digested in nitric acid and analyzed by atomic absorption spectroscopy. Additional information is presented in Sutton et al. (*40*).

Environmental data, particularly dust measurements, were missing from several homes. Dust, paint, and soil lead measures were highly correlated (*40*). For homes with only one absent medium (i.e., dust, paint, or soil) (n = 69 children), we estimated the level of lead in the missing medium from multivariate regression equations derived from the other complete measurements. Housing age was ascertained from county tax assessor data.

Questionnaire. Interviews were administered in English, Spanish, Vietnamese, Cambodian, or Tagalog to the primary caregiver of each child. Questions addressed the child's risk factors for lead exposure, ethnicity, income, education, access to medical care, previous screening for lead poisoning, participation in day care or school, use of vitamins with iron, dwelling renovation, general health status, and a variety of other demographic and health information.

Blood lead and iron status measures. We measured lead levels and iron status in blood samples obtained by venipuncture. Lead and iron status measurements were conducted at the Metabolic Nutrition Laboratory (MNL) at Children's Hospital Oakland. We performed laboratory analysis for blood lead using graphite furnace atomic absorption spectroscopy with a detection limit of 1 µg/dL. MNL participates in the California Department of Health Services Lead Proficiency Testing Program, which, in turn, participates in national proficiency testing programs (*41*). The average percentage differences between measured and true concentrations for 46 external proficiency samples during batch runs was 9.2% for samples < 40 µg/dL. Lead concentrations in the quality control samples were established from the mean of values obtained by five nationally recognized reference laboratories. The coefficient of variation for internal quality control measurements was < 10%. Iron related measures included ferritin, hematocrit (Hct), hemoglobin (Hgb), and mean corpuscular volume (MCV).

Ferritin is an iron-storage protein that maintains sufficient blood iron when dietary intake is inadequate. Ferritin levels may decrease, indicating low iron intake, while other measures of iron status remain normal. Therefore, low ferritin is a highly sensitive and specific indicator of iron deficiency with or without anemia. If ferritin levels are depleted, later signs of iron deficiency may develop, including low hematocrit, hemoglobin, and mean corpuscular volume (*42–44*). Using ferritin as the primary measure of iron status reduces the potential to misclassify low iron status. We chose ferritin levels, *a priori*, as the primary determinant of low iron status. For defining iron deficiency, we used a ferritin cutoff value of ≤ 12 ng/mL (*3,44,45,46*). A secondary analysis used

Table 1. Loadings and eigenvalues for two environmental lead factors derived from principal components analysis of environmental exposure measures.[a]

Components	Factor pattern Factor 1	Factor 2
Ln – Soil lead (µg/g)	0.75	–0.10
Ln – Indoor paint lead (µg/g)	0.58	–0.32
Ln – Outdoor paint lead (µg/g)	0.72	–0.08
Ln – Dust lead level (µg/g)	0.63	–0.49
Ln – Dust lead loading[b] (µg/m^2)	0.30	0.83
House age (years)	0.79	–0.31
Eigenvalues	2.52	1.14

Ln, natural logarithm.
[a]Calculated with SAS Proc Factor, minimum eigenvalue = 1, no rotation (*49,50*). [b]Lead loading = mass of lead per area of floor sampled for house dust, micrograms per square meter.

Table 2. Distribution of demographic characteristics and geometric mean blood lead and ferritin levels by demographic strata.

Covariate	Distribution of total sample n = 382 (%)	Blood lead GM (µg/dL) (± 1 SD)	Ferritin GM[a] (ng/mL) (± 1 SD)
Overall	381 (100)[b]	4.9 (2.5–9.5)	19.1 (8.1–45.1)
Age (years)			
1	59 (15)	5.4 (2.6–11.2)	13.3 (5.3–33.4)
2	82 (22)	4.8 (2.6–9.0)	17.6 (7.5–41.7)
3	102 (27)	5.0 (2.5–9.9)	19.7 (8.2–47.0)
4	74 (19)	4.5 (2.5–7.9)	24.2 (11.8–49.9)
5	64 (17)	4.8 (2.3–20.0)	19.9 (8.4-47.0)
Ethnicity			
Black	95 (25)	5.8 (3.3–10.0)	20.9 (9.2–47.5)
Hispanic	152 (40)	4.4 (2.2–8.6)	16.8 (6.6–42.5)
Asian	68 (18)	5.8 (2.9–11.6)	20.3 (8.4–48.9)
Other[c]	64 (17)	4.1 (2.0–8.4)	22.2 (11.8–41.7)
Sex			
Female	194 (51)	5.1 (2.5–10.3)	16.9 (7.3–39.3)
Male	187 (49)	4.6 (2.5–8.7)	21.5 (9.1–50.9)
SES			
Low	202 (53)	5.4 (2.9–10.1)	18.5 (7.5–46.1)
Medium	116 (30)	4.7 (2.3–9.6)	18.2 (8.1–40.9)
High	63 (17)	3.8 (2.1–7.1)	22.9 (10.8–46.1)
Reported use of vitamins with iron			
Yes	65 (17)	3.8 (2.0–7.2)	17.6 (7.8–39.6)
No	316 (83)	5.1 (2.6–9.9)	19.5 (8.2–46.5)
Time spent in school/day care			
Yes	105 (28)	4.3 (2.3–8.2)	20.7 (9.5–45.2)
No	276 (72)	5.1 (2.9–9.9)	18.5 (7.7–44.7)

GM, geometric mean.
[a]Thirty-five missing ferritin measurements; distribution of reduced samples is very similar to total distribution. [b]One missing blood lead measurement. [c]Predominantly white.

other measures of iron status—Hct, Hgb, and MCV. The age-specific cutoff values to define low iron status were < 33–34% for Hct, < 11–11.2 g/dL for Hgb (*47*), and < 67–73 fL for MCV (*15,48*).

Statistical analyses. We performed all statistical analyses using SAS PC software (*49,50*). Measures of blood and environmental lead and ferritin were log-transformed (*40*).

Initial analyses used simple linear regression and scatter plots to investigate the associations among ferritin, blood lead, and covariates. We then developed multiple linear regression models to assess associations between ferritin and the dependent variable, blood lead, while accounting for potential confounders that affect blood lead and/or iron status measures [age, sex, ethnicity, socioeconomic status (SES), and reported use of vitamins with iron] (*1,14, 15,45*) or were significant in the bivariate analysis. For example, bivariate analyses suggested that attendance in day care or school protected against lead exposure, perhaps because children who spent more time away from their homes may receive less exposure from home contamination. Thus, we controlled for this variable in the regression model.

We performed the above analyses using both a continuous measure of ferritin and a dichotomous measure (≤ 12 or >12 ng/mL). We also examined other measures of iron status (Hgb, Hct, MCV), both individually and as a composite measure, where iron deficiency was assigned if ferritin, Hgb, Hct, or MCV was low (as defined above). Hgb, Hct, and MCV, all later signs of iron deficiency (*13,44*), were not consistently related to blood lead. The results for ferritin and the composite measure of iron status were consistently related to blood lead; of these, ferritin was the best predictor of blood lead. Therefore we report results only for ferritin.

The next steps involved determining whether ferritin status modified the relationship between environmental lead and blood lead. We assigned each child to a high, medium, or low contaminated environment based on a composite measure of contamination. This measure was derived from a principal components analysis (minimum eigenvalue criteria = 1.0) that reduced the six correlated environmental variables (r = 0.15–0.65, p-value = 0.01 or less) (soil, indoor or outdoor paint, dust lead, lead loading, and housing age) to two independent environmental factors.

Table 1 presents the loadings for the variables in each factor. The first factor, Environmental Lead Factor 1, summarizes the largest share of the environmental data (eigenvalue = 2.52) and is primarily a general summary of the environmental lead variables. The second, Environmental Lead Factor 2, (eigenvalue = 1.4) is weighted most heavily by lead loading (the mass of lead per area of floor sample for house dust, micrograms per square meter) and reflects an effect of house dust lead loading that is independent from the overall household lead levels. We calculated contamination scores for each child by multiplying the loadings for each factor by the values of the associated variables and summing. We then assigned tertiles of these scores to high, medium, and low environmental contamination categories for each child.

Next, we conducted simple bivariate analyses to examine trends in blood lead levels between children with low ferritin and normal ferritin levels overall and within each level of environmental lead contamination. Results are presented for individual ethnic groups, all ethnic groups combined, and non-Asians combined. The bivariate analysis confirmed that Asians had a distinctly different relationship between blood lead levels and iron status at each level of environmental contamination. Our final model was run with and without Asians. Final results are presented for non-Asians only.

Finally, we developed a multivariate regression model with the dependent variable blood lead; the independent variables consisted of the covariates, main effects of iron status and environmental category, and an interaction term of these last two variables. We used this model to compute adjusted (least squares) mean blood lead levels for children with low and normal ferritin levels. This strategy allowed us to compare mean blood lead levels within and between environmental lead categories while adjusting for covariates, including age, sex, ethnicity, SES, reported use of vitamins with iron, and whether or not a child spent time in school or day care.

Results

Table 2 presents the study population distribution and blood lead and ferritin levels stratified by major covariates considered in the analysis. Overall, blood lead levels were similar to levels in the U.S. population as a whole at that time (geometric mean = 4.9 µg/dL; maximum = 23 µg/dL). However, 14% of the children exceeded 10 µg/dL, the CDC level of concern. No trends with age were apparent. Blacks and Asians had higher lead levels than Hispanics and whites. Female children also had slightly higher blood lead. Higher SES, reported use of vitamins with iron, and time spent in school or day care were associated with lower lead levels.

The average ferritin level was 19.1 ng/mL (Table 2), with 24% of children having ferritin levels < 12 ng/mL. As expected, ferritin tended to increase with age. Ferritin level was somewhat lower among Hispanics, female children, those with low SES, and those who did not attend school or day care. Paradoxically, ferritin was slightly higher among children with no reported use of vitamins.

Environmental measurements demonstrate significant lead hazards in the homes of many participating children (Table 3). Seventeen percent of soil lead levels were > 500 µg/g, a level associated with significant childhood exposure (*1,2,51*). Exterior paint lead levels were several times higher than interior paint, with 23% and 63% of interior and exterior paint samples, respectively, exceeding 5,000 µg/g, the current Department of Housing and Urban Development action level for abatement (*52*). Seventy-six percent of homes were built before 1950, after which paint lead levels started to decline (*53,54*). The six environmental variables were significantly correlated (r = 0.15–0.65; p-value = 0.01).

Table 4 presents unadjusted geometric mean blood lead levels for children with low ferritin and normal ferritin levels in all ethnic groups. For the population as a whole, the mean blood lead level is slightly higher (by 1 µg/dL) for children with low ferritin levels. This pattern persists within all ethnic groups, except for Asians, where children with normal ferritin levels appear to have higher blood lead levels. Excluding Asian children from the total population increases the difference in blood lead levels between children with low ferritin and those with normal ferritin levels to 1.8 µg/dL.

After adjusting for the potential covariates (ethnicity, sex, age, SES, use of vitamins, and whether or not the child has spent time in school or day care), the geometric mean

Table 3. Descriptive statistics for environmental measurements of lead and housing age.[a]

Medium	No. of homes sampled[b]	No. of children in homes with samples	Mean	± 1 SD	Maximum
Soil	227	375	234[c] µg/g	104–529	2,664
Indoor paint	222	367	1,412[c] µg/g	207–9,611	201,014
Outdoor paint	219	360	8,430[c] µg/g	949–74,892	320,834
Dust concentration	188	312	180[c] µg/g	79–411	3,105
Lead loading	188	312	24[c] µg/g	5–105	886
Housing age	232	361	52[d] (years)	31–73	100

[a]Data from Sutton et al. (*40*). [b]Total number of homes in study: 232. [c]Geometric mean +1 SD. [d]Arithmetic mean ±1 SD.

blood lead levels for non-Asian children with low ferritin and those with normal ferritin were 5.7 and 4.0 µg/dL, respectively (t = 4.0, p-value < 0.01). Including Asian children in the model reduced the magnitude of the difference to 1.0 µg/dL (t = 2.4, p-value = 0.02).

Figure 1 presents the adjusted geometric mean blood lead levels by ferritin status within low, medium, and high lead contamination categories for Environmental Lead Factors (ELF) 1 and 2. We have not included Asian children in these adjusted analyses. Lead levels in children increase with the environmental measures of contamination, as shown in Figure 1. Children with low ferritin levels, regardless of the level of environmental contamination, have higher lead levels than do those with normal ferritin levels. The difference in blood lead levels between those with low and normal ferritin increases as the level of environmental contamination increases. (The mean difference in blood lead levels within each low, medium, and high contamination category for ELF1 = 0.7, 1.9, 3.2 µg/dL, and for ELF2 = 1.7, 0.8, and 2.9 µg/dL, respectively.) The results for both environmental factors are similar. The highest blood lead levels and the largest difference in mean blood lead levels between children with normal and low ferritin are seen in the highest contamination category (3 µg/dL).

Including Asian children in the model tended to reduce the significance and magnitude of the difference in means within each environmental category (about 1 µg/dL) but did not alter the overall pattern. For example, the difference in mean blood lead between low and normal iron-status children in highly contaminated environments was 2.8 µg/dL for ELF1 when Asians were included (p-value = 0.02), but 3.2 µg/dL when Asians were excluded (p-value = 0.01). Excluding the children with estimated environmental data also did not change the results. Finally, because more than one child may have come from the same household, we randomly selected one child from each household to assess possible bias introduced by the lack of independence. Although the statistical significance of some comparisons was reduced because of the smaller sample size, the overall results were not changed (data not shown).

Discussion

Overall, we found that children with iron deficiency, as measured by low ferritin level, had higher blood lead levels than children with normal iron levels. This relationship persisted after we stratified by the level of environmental contamination measured in their homes, with the largest difference in blood lead between iron-deficient and iron-replete children living in the most contaminated environments. These results suggest that inadequate iron status may amplify the effect of lead contamination in the environment by increasing absorption and possibly retention of lead in the body and/or increasing hand-to-mouth or pica behavior and thus lead ingestion (*28,29*).

Our finding is consistent with several studies that have reported higher proportions of children with elevated blood lead among those with low iron levels (*15,16*, *34–36*). Yip and Dallman (*15*) found that the correlation of iron deficiency and blood lead was strongest among the youngest children (1–2 years), weaker in older children, and not significant in adults. This lack of correlation between iron and blood lead in older children (10–18 years) was also reported by Hershko et al. (*37*). The age distribution in our study is limited to young children, who are at highest risk for lead exposure, so our results cannot be generalized to findings for older children.

The relationship of iron status and blood lead varied within ethnic groups in this population, with Asian children having an apparently paradoxical association of sufficient iron status and higher blood lead. We have no clear explanation for this unexpected finding. We have speculated about the possibility of lead-contaminated foods or cooking utensils linking both iron and lead ingestion, but no data are available. The Asian participants in our study were primarily of Southeast Asian origin. It is possible that genetic polymorphisms for δ-aminolevulinic acid dehydratase (ALAD) alleles (*55–58*), or other differences in lead binding proteins could affect blood lead independently of iron status. It is also possible that this finding was caused by chance alone. Additional research is needed to explain intraethnic patterns of lead exposure and iron status.

Our results may be affected by misclassification of iron status or environmental lead exposure. Although low ferritin status is sufficient evidence of iron deficiency (*44*), normal ferritin status does not necessarily indicate iron sufficiency because ferritin is an acute-phase reactant and may be elevated by infection or inflammatory disease (*44*). Thus, some iron deficient children may have been misclassified as iron-replete on the basis of ferritin level, which would bias our results toward the null hypothesis. Similarly, the characterization of environmental lead exposure may have been misclassified because we could not consider a child's behavioral interaction with his or her environment within a given environmental contamination category. The presence of a lead hazard in the home is a necessary but not a sufficient prerequisite for exposure to lead. Children's exposures may vary widely depending on behavior. We also did not consider dietary sources of lead exposure other than possible use of imported pottery and home remedies.

Several factors limit the generalizability of our findings. As a cross-sectional study, it is impossible to determine the temporal pattern of exposure, iron deficiency, and blood lead, so we cannot infer causal relationships between these factors. Additionally, it is possible that iron deficiency is correlated with calcium deficiency, which may also enhance lead

Table 4. Unadjusted blood lead levels by ethnicity and ferritin status.[a]

Ferritin status	Geometric mean blood lead (µg/dL)					
	Black	Hispanic	Asian	Other[b]	Total	Total without Asians
Low ferritin	6.6	5.5	4.6	7.7	5.6	6.0
± 1 SD	4.1–10.6	2.9–10.4	1.9–10.8	3.8–15.5	2.8–11.0	3.3–11.0
n	15	38	17	8	78	61
Normal ferritin	5.3	3.8	6.7	3.8	4.6	4.2
± 1 SD	3.1–9.2	2.0–7.2	3.6–12.3	1.9–7.5	2.4–8.8	2.2–8.0
n	63	93	41	44	241	200

[a]Normal ferritin status: ferritin > 12 ng/mL; low ferritin status: ferritin ≤ 12 ng/mL. [b]Predominantly white.

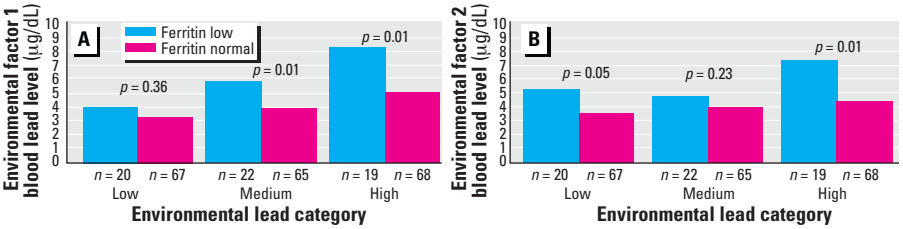

Figure 1. Adjusted geometric mean blood lead levels by ferritin status and environmental lead category: (*A*) Environmental Factor 1; (*B*) Environmental Factor 2. R^2 for full model = 0.23; n = 261 (excludes Asians); p-values for difference of means; ferritin low ≤ 12 ng/mL; ferritin normal > 12 ng/mL. Values adjusted for ethnicity, sex, age, SES, reported use of vitamins with iron, and whether or not the child spent time in school or day care.

absorption (*59–61*). However, the evidence for an inverse relationship between blood lead and calcium intake in the normal physiologic range is uncertain (*62*). Several studies suggest that ingestion of calcium inhibits lead uptake (*35,38,39,59–64*), but the role of chronic calcium deficiency has not been fully elucidated (*62*). Studies of calcium intake and blood lead themselves may be confounded by sociodemographic factors and failure to account for proximate exposure sources.

In summary, we found that iron-deficient children averaged 1–2 μg/dL higher blood lead than children with adequate iron status, with as high as a 3 μg/dL difference for children in the most contaminated environments. By directly controlling for environmental contamination we avoided confounding by the simultaneous presence of sociodemographic lead exposure-risk factors. Because population blood lead levels are log-normally distributed (*10*), small average reductions in lead levels would significantly reduce the proportion of children exceeding 10 μg/dL, the CDC level of concern. Thus, improving iron status in children could, if confirmed, help achieve important public health objectives of reducing blood lead levels below this threshold, particularly for children living in difficult-to-reach contaminated environments. Both iron deficiency and lead exposure disproportionately affect minority, poor, and urban children (*10*). Because iron deficiency has independent effects on cognitive functioning in children that are similar to those of lead poisoning (*1,8,27,65,66*), there should be important prophylactic benefits for children's health and development if organized intensive iron deficiency screening, nutritional counseling, and supplementation were implemented in areas where children are at high risk of both conditions (*67*). Because the relationship between nutritional factors and blood lead is likely to be a complex interaction of nutritional status, individual diurnal and secular nutrient intake patterns, meal frequency, behavior, caregiver ability, and environmental contamination, additional research is urgently needed to validate current hypotheses and quantify the specific benefits of sufficient iron status while accounting for calcium and other major nutrient cations. Because of uncertainties about the benefits of nutritional factors in reducing blood lead (*62*), improved nutritional status must be complemented with removal of lead from children's environments.

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Violence: An Unrecognized Environmental Exposure that May Contribute to Greater Asthma Morbidity in High Risk Inner-City Populations

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In the United States, rising trends in asthma prevalence and severity, which disproportionately impact minorities and the urban poor, have not been fully explained by traditional physical environmental risk factors. Exigencies of inner-city living can increase psychosocial risk factors (e.g., stress) that confer increased asthma morbidity. In the United States, chronic exposure to violence is a unique stressor existing in many high-risk urban neighborhoods. In this paper, we describe a series of cases that exemplify a temporal association between exposure to violence and the precipitation of asthma exacerbations in four urban pediatric patients. In the first three cases, the nature of the exposure is characterized by the proximity to violence, which ranged from direct victimization (through either the threat of physical assault or actual assault) to learning of the death of a peer. The fourth case characterizes a scenario in which a child was exposed to severe parental conflict (i.e., domestic violence) in the hospital setting. Increasingly, studies have begun to explore the effect of living in a violent environment, with a chronic pervasive atmosphere of fear and the perceived or real threat of violence, on health outcomes in population-based studies. Violence exposure may contribute to environmental demands that tax both the individual and the communities in which they live to impact the inner-city asthma burden. At the individual level, intervention strategies aimed to reduce violence exposure, to reduce stress, or to council victims or witnesses to violence may be complementary to more traditional asthma treatment in these populations. Change in policies that address the social, economic, and political factors that contribute to crime and violence in urban America may have broader impact. ***Key words*****: asthma, case series, inner-city, stress, violence.** ***Environ Health Perspect*** **109:1085–1089 (2001). [Online _____]**
http://ehpnet1.niehs.nih.gov/docs/2001/109p1089-1089wright/abstract.html

Case Presentation

We present three cases encountered in the Boston City Hospital Pediatric Allergy–Immunology–Respiratory Clinic and a fourth case seen as an inpatient at Boston City Hospital in which exposure to violence seemed to be the asthma symptom precipitant.

Case 1. Case 1 is a 12-year-old African-American girl with lifelong asthma who has numerous recognized triggers that include pollen, cold air, and exercise. She had presented several times each year to her neighborhood clinic with acute wheezing that responded to nebulized bronchodilator treatment. On initial evaluation in July 1994, her physical exam was notable for allergic rhinitis. Pulmonary function testing showed a mild obstructive defect primarily affecting the small airways: forced vital capacity (FVC), 94%; forced expiratory volume in 1 sec (FEV_1), 79%; and forced expiratory flow rate over the middle 50% of the FVC volume ($FEF_{25\%–75\%}$), 51%. Oral antihistamines, nasal cromolyn, and inhaled steroids were added to her inhaled bronchodilator therapy. In the subsequent month, amoxicillin was begun for sinusitis, and nasal steroids were added to her treatment regimen. After a period of symptom stability she developed increased wheezing in October 1994. Oral prednisone was begun, resulting in rapid improvement to her baseline by the fifth day which was Halloween. On Halloween night, the patient heard gunshots outside of her home in a housing project and shortly thereafter became aware that one of her peers had been fatally shot. She quickly developed recurrent wheezing, slept poorly that night due to respiratory symptoms, and required an extended course of prednisone to control the recurrent asthma exacerbation. Following recovery from this episode, her asthma stabilized.

Case 2. Case 2 is a 15-year-old Hispanic girl who has had severe asthma since infancy and is now enrolled in a college preparatory course in an urban high school. Her history was remarkable because of her need for assisted ventilation with status asthmaticus at the age of 2 years and subsequent every-other-day prednisone therapy up to the age of 5 years. Her currently recognized asthma triggers include exercise, upper respiratory tract infections, and exposure to dust and pets. Allergy skin testing demonstrated sensitivities to several environmental allergens. She was controlled on theophylline, inhaled serevent, flunisolide, nedocromil, oral antihistamines, and regular peak flow monitoring. Typical pulmonary function test results before and after bronchodilator therapy, respectively, in the Pediatric Allergy–Immunology–Respiratory clinic for this patient were FVC, 68% and 100%, FEV_1, 43% and 69%, and $FEF_{25\%–75\%}$, 17% and 31%. During the fall of 1994 she developed increased wheezing on three occasions, which required pulse doses of prednisone. Each episode began on a Sunday evening before the start of a new school week. Inquiry revealed that, at the end of the previous school year, the girl had been attacked on a subway platform by a group of girls. She was physically attacked and her jewelry and book bag, containing her asthma medications, were stolen. In retrospect, Case 2 reported an acute asthma episode immediately after the assault. The patient later identified the assailants to the police and pressed charges against them. Through the fall, the patient encountered her assailants periodically on the subway. She subsequently experienced an asthma flare after a court appearance where she testified against her attackers; during this court appearance, they verbally threatened her. After the sentencing of the assailants, the patient had no further

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R.J.W. received support from NIH training grant HL07427 and the Medical Foundation Deborah Munroe Noonan Memorial Fund.

Received 24 April 2001; accepted 20 July 2001.

documented acute asthma exacerbations for 15 months corresponding to the period of incarceration of her assailants. Over this time course she stopped taking her medications except for an albuterol inhaler as needed and she did not receive follow-up in the Pediatric Allergy-Immunology-Respiratory Clinic. Following the release of the assailants, she again developed severe symptoms requiring two hospitalizations in a 2-month period.

Case 3. Case 3 is a 9-year-old Caucasian girl with asthma since early infancy. The known triggers include exercise, emotional upsets, and upper respiratory tract infections. Allergy skin testing demonstrated sensitivity to many environmental allergens including *Aspergillus*. Sputum cultures have been repeatedly negative for *Aspergillus*, and measured immunoglobulin E (IgE) is 154. Her asthma was managed on inhaled flunisolide, cromolyn, an albuterol inhaler as needed, nasal cromolyn, and diphenhydramine. During the spring of 1994, frequent asthma exacerbations led to a 3-month course of prednisone. Typical pulmonary function test results were as follows: FVC, 90%; FEV_1, 69%; and $FEF_{25\%–75\%}$, 41%. Intensive allergen control measures in the home, including replacing carpeting with linoleum, installing a dehumidifier, restoring crumbling walls, and fumigation, were associated with success in weaning the patient off prednisone and normalization of her spirometry. In October 1994 her daily wheezing returned. It was subsequently revealed that Case 3 had been assaulted on the school bus by an older boy and had reported the incident to teachers. Thereafter the perpetrator's female cousin began to threaten to stab the patient with sharp scissors while they were riding the school bus. The patient finally refused to board the school bus one day for the ride home and subsequently developed wheezing and respiratory distress requiring emergency treatment.

Case 4. Case 4 is a 3-year-old girl admitted to the pediatric intensive care unit (ICU) with an asthma exacerbation in the setting of a viral illness and an exposure to sprayed pesticide 10 days before the onset of symptoms. The patient's initial oxygen saturation was 77% on room air and 89–95% on a 100% non-rebreather face mask. She did not require intubation. Three days into her hospital course, the patient began to show slow clinical improvement on a medical regimen that included continuous nebulized ventolin treatments, intravenous solumedrol, ipratroprium bromide nebulized treatments, and a continuous terbutaline infusion, which was started on the second hospital day.

Case 4's mother stayed with her around the clock. Visits by the patient's father were associated with loud arguments between the parents, which were overheard by the medical staff caring for the patient. On one occasion, a nurse observed the patient's mother slapping the father and then the patient's father pushing and shoving the mother. The health care staff noted that the patient's respiratory rate had increased from 50–60 breaths/min to 80–90 breaths/min during her exposure to these parental encounters. One event documented in the medical record describes the father hitting the mother, causing her to crash into the glass doors of the patient's ICU room. The patient became visibly upset and began screaming. Vital signs documented before the event charted a respiratory rate of 30–34 breaths/min, a heart rate of 145 beats/min, a temperature of 99.4°F, and oxygen saturation of 92% on a 40% face mask. Vital signs documented in the 3–4 hr after the episode showed a clinical decompensation with a respiratory rate of 42–50 breaths/min, a heart rate of 155–180 beats/min, and an initial oxygen saturation of 91% on a 70% face mask. A clinical exam documented decreased air movement and recurrent wheezing associated with the persistent tachypnea and tachycardia.

Discussion

These cases exemplify a temporal association between exposure to violence and the precipitation of asthma exacerbations in four inner-city pediatric patients. Although each patient is vulnerable to a variety of asthma triggers, exposure to violent events seemed to be a common precipitant of asthma symptoms. Notably, Case 2 experienced improvement in her chronic asthma symptoms once the perceived threat of violence was no longer present and deterioration in her respiratory status when that threat reemerged. In Case 4, there was a clear temporal association between witnessing parental conflict and deterioration in the patient's clinical course and vital signs. Because of a raised awareness, we are now inquiring about exposure to violence as an apparent asthma symptom precipitant. Although these cases support a role of exposure to violence and acute exacerbations of established asthma, we should also consider plausible pathways through which living in a violent environment may influence the genesis of asthma.

Asthma is the most common chronic disease of childhood and a leading cause of morbidity in children. In the United States, recent trends of increasing childhood asthma prevalence and morbidity disproportionately affect nonwhite children living in urban areas and children living in poverty (*1–3*). It is not clear that differences in generally known asthma risk factors such as chemical and particulate air pollutants (*4*), environmental and *in utero* tobacco smoke exposure (*5*), viral respiratory infections (*6*), and home allergen exposure (*7*) fully explain these trends. As yet unidentified unique factors may contribute to the higher asthma morbidity and mortality rates seen in inner-city poor minority populations (*8*).

Connections between the health and economic well-being of populations are increasingly seen to be embedded within the larger context of people's lives. It has been proposed that differential exposure to and perception of stress may, in part, explain socioeconomic disparities in health (*9*). Various sociodemographic characteristics (e.g., lower social class, ethnic minority status) may predispose individuals to particular pervasive forms of life stress (*10,11*), and the degree of chronic stress can be significantly influenced by the characteristics of the communities in which people live (*12*). Chronic stress in U.S. urban populations has been conceptualized as neighborhood disadvantage, characterized by the presence of a number of community-level stressors including poverty, unemployment, substandard housing, and high crime/violence rates (*13*). Such physical and social factors can be a source of environmental demands that contribute to stress experienced by populations living in a particular area (*14*).

Studies in minority and lower income populations have shown a high prevalence of children who encounter violence in the inner city. A prevalence study at Boston City Hospital found that 10% of children had witnessed a knifing or shooting before the age of 6 years; 18% had witnessed shoving, kicking, or punching; and 47% had heard gunshots (*15*). In an inner-city cohort in Chicago, Illinois, investigators found that of children between the ages of 7–13, 42% had seen someone shot and 37% had seen someone stabbed (*16*). A survey of urban elementary school children in New Orleans, Louisiana, found that more than 90% had witnessed violent episodes, 70% involving use of weapons (*17*). Although stress is decidedly common and has many causes in our society, the increased prevalence of chronic community violence is a specific and extreme stressor confronting the urban poor.

Violence can be conceptualized as a source of psychological and environmental stress that taxes both the individual and the communities in which they live. Community violence can be considered a pervasive stressor that adds to environmental demands imposed on an already vulnerable population of children and families (*18*). Inner-city populations that experience high rates of exposure to violence are also characterized by high levels of poverty, hopelessness, lack of opportunity, and unemployment (i.e., chronic ongoing stressors). Living in a violent environment is

associated with a chronic pervasive atmosphere of fear and the perceived threat of violence (*19,20*). Children and families living with community violence are likely to view their world and their lives as being out of their control. Facing daily life experiences in an unpredictable or uncontrollable environment predisposes these populations to greater deleterious effects of stress (*21*). Moreover, both the duration and the frequency of experienced stress are important determinants of its impact on health and illness. Variable response to acute challenges (e.g., high frequency of exposure to violence) superimposed on chronic stressors (e.g., other components of neighborhood disadvantage) may have different implications on disease expression (*22*). Events that last a very short time can also have more long-term stress effects through lasting physiologic responses thought to be maintained by recurrent unwanted or "intrusive" thoughts about past events (*23*). Symptoms of post-traumatic stress disorder (PTSD), including flashbacks or recurrent memories of traumatic events, are highly associated with exposure to violence (*24*).

Psychological stress has been associated with the activation of the hypothalamic–pituitary–adrenal (HPA) axis and disturbed regulation of the HPA system.This may best be understood within McEwen el al.'s concept of allostasis, which refers to the ability of the body to achieve stability through change, such that "the autonomic nervous system, the HPA axis, and cardiovascular, metabolic, and immune systems protect the body by responding to internal and external stress" (*25*). The potential cost of such accommodation is conceptualized as allostatic load, which is the wear and tear from chronic overactivity (or underactivity) of the HPA system. With regard to immune function, during a period of acute stress, increased cortisol and catecholamines promote allostasis by influencing cell trafficking and by modulating cytokines, which fight infection (*26*). In contrast, chronic overactivity (or underactivity) of these same mediators may result in allostatic load (i.e., potential immunosuppressive effects when the mediators are chronically secreted or not turned off). Some optimal level of mediators is needed to maintain a functional balance, and the absence of appropriate levels of gluccocorticoids and catecholamines may allow other immune mediators to overreact and increase the risk of inflammatory disorders (*27*). In this framework, violence can be conceptualized as a psychosocial environmental exposure that can "get into the body" and result in biological changes that may contribute to asthma morbidity.

There is a renewed interest in the links between psychological stimuli and asthma (*28,29*). Exposure to violence as a major life stressor may impact on the pathogenesis of asthma and/or contribute to the morbidity of disease by triggering exacerbations through neuroimmunologic mechanisms. Augmented parasympathetic response has been documented after intense or prolonged stress experiences (*30,31*). Increased parasympathetic tone produces increased smooth muscle tone in the lung and thus may mediate emotionally induced bronchoconstriction in asthma (*32*). Cytokines known to be important in inflammatory diseases like asthma may also serve a role in mediating the acute response to physical and emotional stress. Psychosocial stressors can moderate both humoral and cellular immune function (*33,34*). Stressor-linked alterations in the immune system may predispose to respiratory tract infections (*35,36*), which may trigger acute asthma exacerbations. Stress hormones influence immunoglobulin and cytokine expression and thus may increase a genetically predisposed individual's risk of developing asthma. Current knowledge supports the notion that expression of the asthmatic phenotype, as related to the immune response, is modulated by environmental factors that include viral infection, air pollutants, maternal smoking, breast-feeding, and allergen exposure (*37*). Stress may potentiate the allergic response to allergens by increasing the release of inflammatory mediators and the subsequent cascade of inflammatory events characteristic of chronic asthma. That is, violence as a psychosocial stressor may be an "adjuvant" to the asthmatic inflammatory response. Thus, while stress and emotional distress are generally recognized as factors aggravating asthma symptoms in those with existing disease, they may play a role in the genesis of the disease as well (*29*).

Preliminary empirical evidence suggests that exposure to violence may contribute to the burden of asthma morbidity on the inner-city poor. In a cohort study in Boston, Wright et al. (*38*) retrospectively ascertained lifetime exposure to violence through a parental-report interview questionnaire administered to 416 caregivers and their children who are being followed longitudinally for respiratory health outcomes, including asthma. Preliminary analyses suggest a link between higher lifetime exposure to community violence and an increased risk of asthma and wheeze syndromes and prescription bronchodilator use.

Violence exposure may ameliorate resources needed to manage and cope with chronic asthma. Exposure to community violence (and other determinants of neighborhood disadvantage) may operate through effects on impulse control, risk-taking behavior, and the adoption of coping behaviors such as smoking, thus leading to increased exposure to a known environmental asthma trigger (*39*). Smoking can be conceptualized, at the individual level, as a strategy to cope with negative affect or stress (*40,41*). Neighborhood effects on health behaviors such as smoking have also been demonstrated (*42,43*). For example, evidence from the 1987 General Social Survey (*44*) suggests that stress may be one factor promoting increased prevalence of smoking in African-American communities. Romano et al. (*45*) surveyed 1,137 African-American households and found that the strongest predictor of smoking was a report of high-level stress, represented by a "hassles" index. The "hassles" index was an abbreviated 10-item scale based on items chosen to represent a dimension that community residents involved in the project perceived to be especially relevant. Notably, among the items were neighborhood level factors including being concerned about violence or living in an unsafe area.

Community-level characteristics such as increased prevalence of violence may influence an individual's behavior, resulting in increased exposure to other known environmental risk factors for asthma. Parents in high-violence communities may restrict their children's outdoor activities. In the same Boston pediatric cohort discussed above, parental reports of keeping children indoors primarily because of fear of neighborhood violence was related to increased risk of wheeze and physician's's diagnosis of asthma prior to the age of 2 years (*46*). Reasonable hypotheses as to why this association was seen may include the following. The child who is kept indoors may become deconditioned, experiencing shortness of breath with decreasing levels of exertion. An increased sedentary lifestyle may be linked to obesity in children. Recent studies have linked obesity to asthma (*47,48*), and studies suggest that obesity has increased among families living in poverty in the United States (*49*). Also, children who are kept indoors may be exposed for longer periods to indoor aeroallergens and have an increased likelihood of sensitization and allergic symptoms in response to dust mite, pet, roach, and rodent allergens. Parents who are worried about their children's safety in their neighborhood because of crime may keep their children indoors and otherwise restrict their social behavior; thus each child's ability to develop support networks may be compromised (i.e., exposure to violence may lead to diminished stress-buffering factors such as social networks) (*50*). Psychopathology (e.g., PTSD, depression) influenced by life stress and chronic exposure to violence may also prevent the child from forming relationships that are necessary to promote normal social development. Fear of crime fosters a distrust of

others and can contribute to social isolation (*51*). It is clear that violence is related to factors that limit formation of social networks. These additional supports may be especially important to health and well-being in high-risk urban populations faced with cumulative effects of many other ecologic stressors (i.e., poverty, low education, poor housing).

Coping with a violent environment may affect compliance with therapy and medical follow-up for asthma. Fong (*52*) discussed the impact of violence on the management of hypertensive urban African Americans, underscoring violence as a perceived barrier to keeping appointments and following prescribed exercise programs. Fear of making a trip across town to a pharmacy or medical facility or adhering to a prescribed walking program as a result of prior victimization or a perceived threat of violence may be a barrier to compliance. This may lead to lapses in use of prophylactic medication, delayed intervention, and consequently greater morbidity. Adolescents who witness violence are more likely to develop a foreshortened sense of the future (*53*) and thus a fatalistic outlook that may undermine their ability to invest in the future by complying with a chronic asthma treatment regimen. Other barriers to adherence to a prescribed asthma regimen may include the lack of a community pharmacy open 24 hr/day. Pharmacies may be reluctant to remain open 24 hr/day in poor communities, especially when violence is a concern. Violence can indirectly affect access to medical care by diverting limited funds away from primary care and specialty clinics, including those caring for asthmatics (*54,55*).

Exposure to violence may affect asthma management when increased family dysfunction impedes development of appropriate coping strategies necessary to facilitate improved quality of care for the asthmatic child. Dysfunctional patterns are common in homes of children with asthma and may be precipitated by anxiety experienced around asthmatic attacks (*56*). Family dysfunction has been related to increased asthma morbidity and mortality (*57,58*). The level of stress in the home of an asthmatic child is likely to increase as parents attempt to balance the child's need for activity and independence with their concerns about avoiding allergen- or exercise-induced symptoms and maintaining adherence to a pharmacologic regimen. Likewise, stress and anxiety may be compounded in families who are also faced with the real or perceived threat of violence or injury in the child's home, neighborhood, or school, which leads to greater dysfunction. Parents who have experienced violence, or whose children have had such experiences, may develop depression or PTSD, which impairs their ability to supervise and respond to their children. This reduction in parenting capacity may undermine an adult's ability to coordinate a child's ongoing asthma care.

Conclusions

Exigencies of inner-city living, such as coping with the high prevalence of exposure to violence, may increase psychosocial risk factors, which in turn may confer increased asthma morbidity on high-risk urban populations. High crime rates, and thus the real or perceived threat of violence, are specific aspects of the inner-city environment that may impact psychologic functioning as well as health-promoting and health care-seeking behaviors of the inhabitants (*59*). More research is needed to examine the public health impact of children and their families living with violence. Systematic exploration of an association between violence (an urban stressor) and asthma throughout childhood may help us to understand the rise in asthma prevalence, severity, and medical care use as well as to further our understanding of its disproportionate occurrence in poor urban children in this country. We present these cases to alert clinicians and researchers to a potential risk factor for increased asthma morbidity that has not previously been recognized.

Increasingly, pediatricians are being asked to manage chronic childhood illness in the context of complicated family and community environments that clearly impact disease management. Pediatricians have long recognized the impact of violence on the health and well-being of children and have been expanding efforts to increase response to exposure to violence as a health care issue in the clinical setting (*60*). The identification of exposure to violence as a trigger of asthma exacerbations may alert health professionals caring for asthmatics in the inner-city setting to inquire about patient's exposure among other known triggers. Secondary intervention strategies designed to reduce exposure to violence or to facilitate positive coping mechanisms for individual patients may obviate the need for more aggressive and costly pharmacologic therapies for asthma with potential side effects. For example, referral to a stress reduction program or to programs that provide counseling for children who have witnessed or experienced violence (*61*) may be helpful. In our experience, it is unlikely that the child's asthma control can be improved unless such psychosocial issues are also addressed.

Primary prevention at the population or neighborhood level should also be considered. Social cohesion and social capital are strongly correlated with rates of violent crime within neighborhoods (*62*). Research suggests that crime is most prevalent in societies that permit large disparities in the material standards of living of its citizens, which in turn are created by broad-scale societal and political factors (*63,64*). Emerging evidence underscores the need for policy makers to pay increased attention to political and economic forces that result in further marginalization of minority populations in the inner city and contribute to the growing income gap between the rich and the poor in this country (*65*). Policies aimed at improvements in life opportunities and living conditions may increase social cohesion and decrease violence in the inner cities. Social cohesion may influence the health behaviors of neighborhood residents by promoting diffusion of health information or increasing the adoption of healthy behaviors through exerting social control over smoking. Improved neighborhood social capital may impact health through increased access to local services and amenities (e.g., safe transportation, pharmacy availability). It is unlikely that the health problems of disadvantaged populations can be solved unless we try to understand the potential role of unique environmental stressors such as violence exposure.

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