

A Novel Endocrine-Disrupting Agent in Corn with Mitogenic Activity in Human Breast and Prostatic Cancer Cells

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Housing adult rats on ground corncob bedding impedes male and female mating behavior and causes acyclicity in females. The suppressive effects on ovarian cyclicity are mimicked by a mitogenic agent purified from the ground corncob bedding material (corn mitogen; CM), which stimulates the proliferation of estrogen receptor (ER)-positive (MCF-7 cells) and ER-negative (MDA-MD-231 cells) breast cancer cells. Purified CM does not compete for [³H]estradiol binding to ER or nuclear type II sites, and its effects on MCF-7 breast cancer cell proliferation are not blocked by the antiestrogen ICI-182,780. These results suggest that the active component is unlikely to be a phytoestrogen, bioflavonoid, mycotoxin, or other known endocrine-disrupting agent that modifies cell growth via ER or type II [³H]estradiol binding sites. CM also stimulates the proliferation of PC-3 human prostatic cancer cells *in vitro*, and the growth rate of PC-3 cell xenografts is accelerated in nude male mice housed on ground corncob as opposed to pure cellulose bedding. Consequently, this endocrine-disrupting agent in ground corncob bedding may influence behavioral and physiologic reproductive response profiles and malignant cell proliferation in experimental animals. Fresh corn (kernels and cob) or corn tortillas also contain CM, indicating that human exposure is likely; consequently, CM and/or related mitogens in corn products may influence human health and development. **Key words:** breast cancer, corncob bedding, corn mitogen, endocrine disruptor, estrogen receptor. *Environ Health Perspect* 110:169–177 (2002). [Online 17 January 2002]

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We have known for decades that environmentally derived phytoestrogens and mycotoxins profoundly affect reproductive function. These substances, which most often influence estrogen receptor (ER)-regulated pathways, stimulate cellular differentiation and proliferation of the mammary gland (1) and uterine growth and DNA synthesis in rodents (2). Consequently, it is not surprising that consumption of soy-supplemented diets is associated with the low-level stimulation of vaginal cornification in postmenopausal women (3). On this basis, some investigators have suggested that phytoestrogens with impeded estrogenic activity may actually reduce the incidence of breast and prostate cancer in humans by competitively blocking the effects of more active physiologic estrogens such as estradiol (4,5). Although this hypothesis appears logical on the surface, it is well documented that sustained exposure to impeded estrogens (estriol) or antiestrogens (tamoxifen) with mild estrogenic activity causes mammary cancer in experimental animals (6) and endometrial cancer in women (7). Thus, “impeded” estrogenic agents may be carcinogenic when exposure is sustained, and their impact on human health and development may be substantially underestimated. Consequently, environmentally derived endocrine disruptors with mitogenic activity may represent a significant health problem if exposure is chronic.

The studies described in this article concern a mitogenic agent isolated from ground corncob animal bedding (corn mitogen; CM), from commercially available fresh corn (kernels or cob), or from corn tortillas which blocks male and female reproductive behavior and also causes persistent metestrus in adult female rats. CM does not appear to be a classical phytoestrogen (8) or mycotoxin such as zearalenone (9,10) because it does not bind to ER and is able to stimulate proliferation of ER-positive or ER-negative human breast cancer cells. Highly purified preparations of CM also do not compete for [³H]estradiol binding to nuclear type II sites in rat uterine preparations, as do other bioflavonoids or related metabolites (11,12), further suggesting that the mitogenic activity of CM is mediated through nonestrogen-regulated pathways. CM also stimulates the growth and proliferation of PC-3 human prostatic cancer cells *in vitro*. Subcutaneous PC-3 cell xenografts grow more rapidly in athymic nude mice housed on ground corncob bedding than in mice housed on pure cellulose. Therefore, exposure to corncob bedding materials or corn food products may influence experimental results in studies involving male and female reproductive function and/or breast and prostatic cancer cell proliferation. Because corn represents a major human food source, sustained exposure to CM could affect human health, development, and reproductive function as well.

Methods and Materials

Animals and treatment. The adult male and female Sprague-Dawley rats used for these studies were purchased from Harlan (Houston, TX). For some experiments, adult female rats were ovariectomized under metofane anesthesia and rested for 14 days before the study. Animals were housed in suspended wire cages, with hardwood chips (Sani-chips; P.J. Murphy Forest Product Corporation, Montville, NJ) or ground corncob (Bedocob; Green Products Company, Conrad, IA) bedding as described for the individual studies below. Standard lighting conditions (12-hr light–dark cycle, lights on at 0600 hr) were used unless specified otherwise in the text and figure legends. Food and water were provided *ad libitum*. We used BALB/c nu/nu athymic nude mice (Harlan), to assess the effects of cellulose or corncob bedding material on the growth of PC-3 human prostatic cancer cell xenografts. These animals were housed in cages containing the autoclaved purified cellulose or ground corncob bedding in the pathogen-free barrier facility at Baylor College of Medicine. Autoclaving did not modify the CM activity in the corncob bedding material for studies with nude mice. Mice were housed under constant lighting conditions, and sterilized food and water were provided *ad libitum*.

All animals were housed in Association for Assessment and Accreditation of Laboratory Animal Care–approved animal facilities and received the highest standard of humane care in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* (13). Ovariectomies were performed under metofane anesthesia using aseptic

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techniques in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* as approved by the institutional Committee on Animal Care and Use. In some studies, animals were anesthetized with metofane or a combination of ketamine, acepromazine, and xylazine and killed by cervical dislocation in accordance with the aforementioned protocols.

Effects of housing conditions on uterotrophic response. Intact or ovariectomized 60-day-old female rats (8–10 per group) were housed for 14 days in wire cages, on hardwood chips, or on ground corncob bedding. At the termination of the study, the animals were killed and uteri were removed, stripped of extraneous tissue, rinsed in chilled saline, blotted, and weighed. We determined uterine wet weight or DNA content as previously described (14). Data were expressed as the mean \pm SE for each experimental group and analyzed statistically with Instat (GraphPad Software, San Diego, CA), using analysis of variance (ANOVA) and a suitable multiple range test on the treatment means.

Effects of housing conditions on estrous cycles in female rats. We housed 60-day-old female rats in wire cages and collected daily vaginal smears for 2–3 weeks to establish a baseline index of cyclicity for each female. Smears were stained with Giemsa and scored for proestrus, estrus, metestrus, or diestrus according to classical vaginal smearing technique (15). Data were expressed as the percentage of animals displaying normal estrous cycles. The daily pattern of cyclicity was established for each female during this 2- to 3-week period. After these preliminary studies, groups of these animals (10 per treatment group) were housed in wire cages or on corncob bedding for either 2 or 5 weeks. Daily vaginal smears were taken during this period, and estrous cycles were recorded. In addition, a second group of 10 rats were housed in wire cages for 14 days, moved to corncob bedding for 14 days, and returned to wire cages for 14 days. Daily vaginal smears were collected throughout this 52-day period, and bedding effects on the numbers of animals displaying normal 4- to 5-day estrous cycles were determined.

In some studies, we assessed the effects of orally administered CM extracts on estrous cycles in adult female Sprague-Dawley rats housed in wire cages. Daily vaginal smears were taken for 6 weeks to establish that all animals displayed normal 4- to 5-day cycles before treatment. During week 7, the animals were segregated into treatment groups (10 rats/group). The groups were given 2% Tween-80–tap water vehicle (controls), a low dose (0.2 mL/400 mL), or a high dose (2.0 mL/400 mL) of the purified CM extract

[80% methanol Spice (Rainin, Woburn, MA) cartridge eluate reconstituted as described below] dissolved in this vehicle. Vaginal smears were collected daily throughout the 2- to 3-week treatment period. The ovarian cycles of the vehicle controls did not differ from those of animals maintained on tap water (data not shown), and thus the vehicle had no significant effect on the estrous cycles of control animals.

Assessment of reproductive behavior in male rats. Intact Harlan-Sprague-Dawley male rats (10 per group) were housed in wire cages, and their sexual performance in the presence of sexually receptive females was monitored during the dark phase of the reversed light cycle. Ovariectomized-steroid hormone-primed females of the same strain were used as stimulus animals. These females were brought into behavioral estrus by a subcutaneous (sc) priming injection of 2 μ g of estradiol benzoate (EB) in sesame oil 48 hr before receiving 100 μ g of progesterone (sc). We evaluated male sexual behavior by examining the number of mounts, intromissions, ejaculations, ejaculation latencies (in seconds) and grooming frequencies in a 30-min test period with each sexually receptive female as per established procedures (16). Sexual behavior in the presence of sexually receptive (estrogen and progesterone primed) ovariectomized rats housed in wire cages was also evaluated for adult male proven breeders separately housed on wood chips (Sani-Chips) or ground corncob bedding for 7–10 days before exposure to the females. The numbers of mounts, intromissions, ejaculations, ejaculation latencies in seconds, or grooming frequencies observed during a 30-min test period with each sexually receptive female was scored.

Assessment of mating behavior (lordosis) in female rats. We also evaluated the effect of CM on the mating behavior of female rats to determine whether this mitogenic agent altered their receptive responses to males (lordosis). For these studies, ovariectomized rats (10 per group) were housed in hanging metal cages (controls) or on ground corncob bedding (treated animals) and maintained on reverse light–dark cycles with lights off at 1200 hr and on at 2400 hr. These females were primed with physiologic doses of estradiol benzoate (2 μ g) followed by progesterone (100 μ g) 48 hr later. The animals in both groups were tested for lordosis response in the presence of male rats during the dark phase of the cycle, following published protocols (17,18). Lordosis is characterized by classical back-arching reflex by the female upon mounting by the male for a total of 10 mounts. This was scored and represented as lordosis quotient (LQ = no. of lordoses/no. of mounts \times 100) and determined for the animals housed on wire or corncob bedding.

Isolation of CM activity from corncob bedding or fresh corn. The methodology for the isolation and purification of CM was developed in our laboratory during the course of this investigation. Methanol and ethyl acetate were high-performance liquid chromatography (HPLC) grade, and 100% ethanol was glass redistilled before use. Approximately 200 g of ground corncob bedding (or corn kernels or cob from one ear of corn) were extracted at 70°C for 2 hr in 600 mL of HPLC-grade methanol. The extract was filtered through two layers of Whatman #1 filter paper and concentrated under vacuum at 70°C to a final volume of approximately 75 mL. Aliquots of this material were dried under nitrogen at 70°C and redissolved in 100% ethanol for further purification or evaluation for mitogenic activity. When concentrated to ~75 mL, 2–4 μ L of this extract stimulates MCF-7 human breast cancer cell proliferation in a manner equivalent to the response obtained with 1 nM estradiol. MDA-MD-231 cells are more sensitive to CM than MCF-7 cells. Appropriate control experiments have shown that CM in our preparations is derived from corn kernels and/or cob and is not a solvent contaminant. CM activity is also readily extracted from ground corncob bedding with ethyl acetate (ETAC) (not shown). For the ETAC extractions, approximately 200 g ground corncob bedding was extracted at 22°C for 24–48 hr in 600 mL of HPLC-grade ETAC, filtered, and concentrated as described above for methanol extracts. Either extraction procedure generated approximately equivalent quantities of chromatographically indistinguishable CM, although the ETAC extracts typically contained lower quantities of polar contaminants.

In one series of experiments, 12 oz commercially obtained corn tortillas were extracted with ~400 mL of methanol as described above for the ground corncob bedding. This tortilla extract was diluted to 20% methanol with milliQ-water (Millipore, Austin, TX), loaded onto a C18 mini-column as described below, washed with 60% methanol, and eluted with 80% methanol. An aliquot of the 80% methanol extract (100 μ L) was taken to dryness under nitrogen at 70°C and redissolved in 2 mL ethanol. Aliquots (2–10 μ L) of this partially purified preparation were added to MCF-7 breast cancer cells to assess effects on cellular proliferation as described below.

Chromatography of CM on C18 reverse-phase mini-columns. The mitogenic agent in CM preparations is quantitatively recovered from Spice C18 reverse-phase cartridges, BOND ELUTE C18 mini-columns (Rainin/Varian, Walnut Creek, CA), or Sep-Pak C18 reverse-phase cartridges

(Waters, Milford, MA) equilibrated in water containing < 35% methanol or ethanol. To further explore the use of C18 reverse-phase resin for CM purification, we diluted aliquots of the corncob bedding methanol extract (CM) with HPLC-grade water to a final methanol concentration of approximately 10% and loaded them onto BOND ELUTE mini-columns (500 mg resin). The pass-through and column wash with 10% methanol were separately collected, as were the sequential eluates with 10 mL volumes of 20%, 40%, 60%, 80%, and 100% methanol. Each eluate was taken to dryness under nitrogen at 70°C and redissolved in 2.0 mL of 100% ethanol before addition to the cell cultures. Elution of the mini-columns with 80% methanol (or ethanol) resulted in complete recovery of the mitogenic activity from corncob bedding or from fresh corn kernels and cob. As anticipated, we subsequently demonstrated that the elution behavior of the mitogenic activity from C18 mini-columns and cartridges with 20–100% ethanol mirrored that observed with methanol. This simplified the exchange of solvents used for extraction (methanol) to those more suitable for addition to cell cultures (100% ethanol) or [³H]estradiol binding assays. The purified material was simply diluted to < 35% methanol with milli-Q water, collected on C18 mini-columns equilibrated in milli-Q water, washed with 35% ethanol in milli-Q water, and collected with 100% ethanol. The ethanol eluate of the mitogenic agent could be directly added to cultured cells.

Reverse-phase HPLC of CM in ground corncob extracts. The data generated on the C18 cartridges and mini-columns suggested that chromatography on C18 reverse-phase HPLC columns may be used to further purify CM from these various corn extracts. HPLC was performed on a Beckman Gradient Liquid Chromatography System (Beckman Instruments, Fullerton, CA) equipped with single-channel fixed wavelength detector (280 nm). Partially purified CM preparations (80% methanol fraction eluted from the C18 mini-columns) were injected onto a Waters μ Bondapak C18 analytic HPLC column eluted isocratically with water:methanol (30:70) at a flow rate of 1 mL/min. Fractions were collected and assayed for mitogenic activity in MCF-7 cell cultures on 24-well plates as described below. The mitogenic activity coeluted with a single peak of UV-absorbing material, suggesting that these preparations were relatively clean and fairly homogeneous with respect to the mitogenic activity.

Assessment of mitogenic activity of CM preparations in human breast cancer cells. We assessed the effects of the various CM preparations and chromatography fractions on the proliferation of human breast

(MCF-7 or MDA-MD-231) cells by routine procedures in our laboratory (12,19,20). For these studies, the CM preparations and column fractions were dried under nitrogen at 60–70°C and redissolved in a known volume (100–500 μ L) of 100% ethanol or collected from C18 mini-columns in 100% ethanol as described above. Aliquots (1–10 μ L) of each sample or equivalent quantities of 100% ethanol (controls) were added to quadruplicate wells (24-well plates seeded with 30,000 cells/well) of MCF-7 or MDA-MD-231 cells grown in 1 mL phenol red-free medium containing 10% charcoal-stripped, sulfatase-treated, fetal calf serum (21). We added estradiol (1 nM) and ICI-182,780 (ICI; 10 nM) to the wells in 1–10 μ L ethanol where indicated. We determined cell numbers (hemocytometer counts) 4–6 days after treatment (22,23). In some experiments, exponentially growing MCF-7 cells were treated with 1 nM estradiol (E₂), 10 nM ICI-182,780, and/or 10 μ L highly purified ground corncob bedding extract (80% eluate from Spice C18 Cartridge), and we determined the cell number 6 days after treatment. We calculated the results from quadruplicate wells for each treatment group as a percent of control (cells grown in the presence of ethanol vehicle), and plotted the data as the mean \pm SE to assess treatment effects on cellular proliferation. Data were analyzed statistically via ANOVA and a suitable multiple range test on the treatment means using Instat as described below.

Interaction of CM with ER and type II sites in rat uterine cytosol and nuclear preparations. We evaluated the ability of CM to competitively inhibit [³H]estradiol binding to ER or nuclear type II sites (14,24). It is difficult to cleanly discriminate between [³H]estradiol binding to ER and type II sites, even with full saturation analysis over a wide range of [³H]estradiol concentrations. Therefore, we used assay procedures that separately measure either ER or type II sites with single saturating [³H]estradiol concentrations (10 nM or ER and 30 nM for type II sites) as previously described in detail (24). For competition studies with ER, uteri from adult ovariectomized rats were homogenized in TED buffer (10 mM Tris, 1.5 mM EDTA, 10 mM dithiothreitol, pH 7.4; this reducing agent blocks type II binding sites) and aliquots of the high-speed (39,000 $g \times 30$ min) supernatant (cytosol) were incubated (30°C for 30 min) in the presence of 10 nM [³H]estradiol \pm various aliquots of crude or HPLC purified CM (or 300-fold excess diethylstilbestrol) under conditions that measure ER. Following incubation, bound and free steroid was separated by hydroxyapatite adsorption as previously

described (14). Type II sites do not bind [³H]estradiol in the presence of dithiothreitol, and ER is quantified without interference from this secondary binding component (23). We did not discriminate between CM binding to ER α or ER β (25,26) in these cytosol preparations.

CM competition for nuclear type II sites was performed with uterine nuclear fractions from estradiol-implanted rats because nuclear type II sites are stimulated 30-fold in the ovariectomized rat uterus by estradiol (14). Uterine nuclear suspensions prepared from estradiol-implanted rats were incubated in the presence of 30 nM [³H]estradiol \pm various aliquots of crude or HPLC-purified CM (or 300-fold excess luteolin) under conditions (4°C for 60 min) that do not measure occupied ER (24). Thus, type II sites can be measured with a single saturating [³H]estradiol concentration without interference from ER. Results from these binding studies are expressed as percent [³H]estradiol bound, where 100% (control) represents approximately 10,000 cpm for ER and 25,000 cpm per assay tube for type II sites.

CM effects on human prostatic cancer cells in vitro and in vivo. We used PC-3 prostate cancer cells (ATCC) to assess CM effects on the growth and proliferation of prostate cancer cells *in vitro* and *in vivo*. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM)-F12 containing 10% fetal calf serum and 1% penicillin-streptomycin as described previously by our laboratory (11,23,27). To assess CM effects on cell proliferation, PC-3 cells were seeded in 96-well plates (1,000 cells/well) containing 200 μ L DMEM-F12 medium and allowed to attach for 24 hr. After attachment (day 0), the medium was replaced with fresh medium, and the cells were treated with 1–10 μ L of ethanol (controls) or CM (80% methanol C18 fraction) reconstituted in ethanol. The cells were grown in the presence of CM for 6 days and medium, and the CM or vehicle was replaced every 48 hr. We determined cell number by hemocytometer counts or via the MTT assay (28). The methyl thiazoyl tetrazolium (MTT) assay routinely duplicates results determined on the basis of DNA content per well or hemocytometer counts (not shown). Cellular proliferation can be readily quantified with the MTT assay procedure in 96-well plates with a microplate reader at 570 nm.

We also assessed the growth rate of subcutaneous PC-3 cell xenografts in Harlan adult male BALB/c nu/nu athymic nude mice (6–10 mice per group) housed on pure cellulose or ground corncob bedding. After housing on the appropriate bedding material for 21 days, these mice were injected subcutaneously in the flank with PC-3 cells (3.5×10^6 cells in 0.15 mL DMEM-F12

medium) as described previously (20). Approximately 3 weeks after PC-3 cell injection, subcutaneous solid tumors ~0.3 cm × 0.3 cm (length × width) were detected in > 99% of the animals. This was designated day zero, and tumor size (length × width) was monitored for the next 8 days. Results were expressed as the percent day 0 control throughout the course of the study. These tumors grew rapidly, and the animals were sacrificed before the PC-3 cell xenografts reached 10% of their body weights.

Statistical analyses. Data, where appropriate, were analyzed statistically with Instat (GraphPad Software) using a one-way ANOVA and an appropriate multiple range test on the treatment means. Values in cell proliferation assays represent the means ± the SE for 12–16 independent observations (three replicates of assays using quadruplicate wells per treatment group). Uterine weights values typically represent data collected from 8–10 animals per treatment group (for 2–3 replicate experiments) and tumor growth values were generated from tumors growing in 6–10 mice per treatment group.

Results

Effects of bedding on uterotrophic response, reproductive behavior, and ovarian cyclicity.

The search for CM was prompted by the fact that the breeding efficiency of our rats housed on ground corncob bedding was substantially reduced compared to that of rats housed on woodchips. This suggested that the corncob bedding contained an endocrine-disrupting agent that was not present in the hardwood chips or perhaps other bedding materials. To

evaluate these possibilities, we assessed the effects of housing conditions on uterotrophic response (uterine weight and DNA content) in the rat. This is a classic bioassay for estrogenic activity and/or antiestrogenic activity (29). When adult, ovariectomized rats were housed on woodchips (Figure 1), uterine wet weight ($p < 0.01$) and DNA content doubled ($p < 0.01$) relative to those of controls (adult-ovariectomized rats housed in wire cages). This appeared to be a classic estrogenic response, which reversed when the animals housed on the woodchips were returned to wire cages (data not shown). Thus, it appears that the woodchips contained an estrogenic component that reversibly modulated uterine

growth, and we used this response for comparison to responses obtained in animals housed on the corncob bedding. This is not surprising in view of the fact that woodchips are likely to contain phytoestrogens known to stimulate uterotrophic response in the rat (2,30,31).

Conversely, housing intact adult female rats on ground corncob bedding decreased ($p < 0.01$ or $p < 0.001$) uterine wet weight (Figure 2A) and DNA content (Figure 2B) relative to wire-housed controls or in animals housed on the woodchips (Figure 1). Approximately 90% of the animals housed in wire cages for 2 weeks or 5 weeks displayed normal estrous cycles. Marked reductions

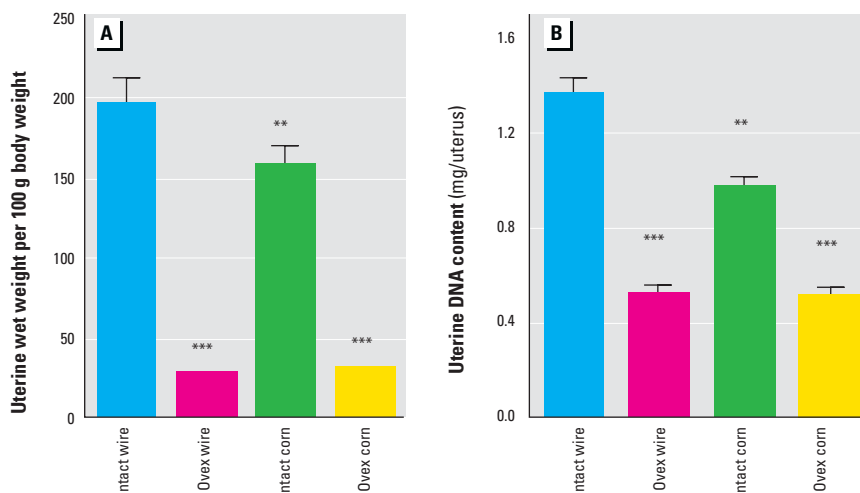


Figure 2. Effects of housing on corncob bedding on wet weight and DNA content of the rat uterus. Adult intact or ovariectomized (Ovex) rats were housed in wire cages or on corncob bedding for 14 days before uterine wet weight (A) and DNA content (B) were determined. Housing adult, ovariectomized animals on ground corncob bedding significantly reduced (** $p < 0.01$ or *** $p < 0.001$) uterine wet weight (A) or DNA content (B) compared to intact animals housed on wire.

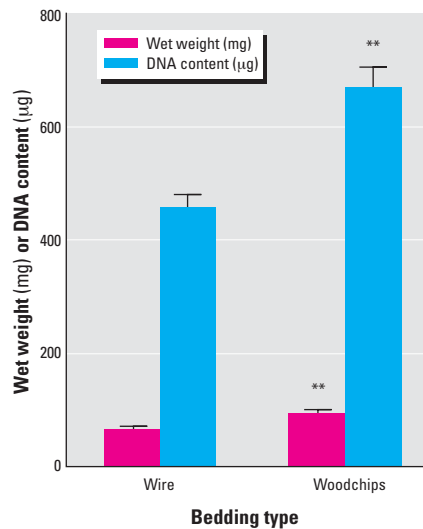


Figure 1. Effects of housing conditions on wet weight and DNA content of the rat uterus. Adult, ovariectomized rats were housed in wire cages or on woodchips. Uterine wet weight (µg/uterus) and DNA content (µg/uterus) were higher (** $p < 0.01$) in animals housed on woodchips than in wire cages.

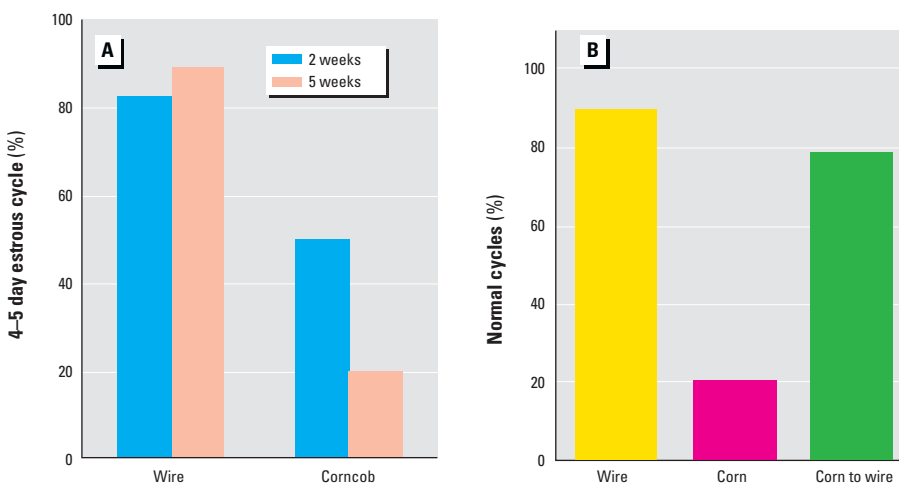


Figure 3. Effects of housing conditions on the estrous cycle in adult Sprague-Dawley rats. Sixty-day-old female rats (8–10 per group) were housed in wire cages or on corncob bedding for either 2 or 5 weeks, and daily estrous cycles were recorded (A). A second group of 10 rats (B) were housed in wire cages for 14 days, moved to corncob bedding for 14 days, and returned to wire cages for 14 days. Daily vaginal smears were collected throughout this period. Exposure to the corncob bedding disrupted estrous cycles and caused persistent metestrus in adult female rats, and this effect was reversible when the animals were returned to the wire cages.

(50–80%) in cyclicity were noted in animals housed on corncob bedding (Figure 3A). The vaginal smears from these animals reflected a state of persistent metestrus (15), and the acyclicity was associated with a substantial decrease (> 90%) in lordosis response (Figure 4A). Normal cyclicity (Figure 3B) and lordosis response (data not shown) resumed when these animals were returned to wire housing. That the effects of the bedding extract on uterine wet weight and DNA content were observed in intact but not in ovariectomized animals (Figure 2) suggests that an ovarian component may be involved in this response. Such an effect could involve the modulation of gonadotrophin release by factors derived from corncob bedding and downstream effects on ovarian steroid biosynthesis. Similarly, males housed on ground corncob bedding also displayed substantial reductions (Figure 4B) in mounting behavior (> 98%), intromission (> 97%), ejaculation (> 98%), ejaculation latency (> 79%), and grooming frequency (~75%) when placed with breeder females, which could be linked to the hypothalamic–pituitary–testicular axis.

Effects of corncob extracts on breast cancer cell proliferation. Because exposure to ground corncob bedding induced persistent metestrus in rats, we suspected that an agent derived from the corncob bedding was modulating estrogen-regulated pathways. Therefore, we used the MCF-7 human breast cancer cell line to monitor extracts of ground cob bedding (CM) for estrogenic or antiestrogenic activity. MCF-7 human breast cancer cells contain ER and proliferate in response to estrogen. However, this proliferative response is ablated by antiestrogens such as ICI-182,780 (32). Conversely, MDA-MD 231 cells lack functional ER and do not respond to estrogens and antiestrogens in a classical manner. These cells were used as ER-negative controls (32–35). The active agent (CM) was extracted from ground corncob bedding at 70°C with methanol, dried under vacuum, and reconstituted in 100% ethanol. This CM preparation was added to exponentially growing cultures of MCF-7 or MDA-MD-231 cells, and equivalent quantities of 100% ethanol were added to appropriate controls. Treatment of the cells with various quantities (2–10 μ L) of the nondiluted extract produced a classic dose–response curve in MCF-7 cells. Lower doses of the extract stimulated MCF-7 cell proliferation, and the magnitude of the stimulatory response was equivalent to that obtained with 1 nM estradiol (Figure 5A). MCF-7 cell proliferation was inhibited ($p < 0.01$) by higher doses of the extract, and this response may have been augmented by coincidental treatment with estradiol. Whether this inhibitory response resulted

from an additive or synergistic interaction to CM and estradiol remains to be resolved. Surprisingly, the lower concentrations of the corncob bedding extract failed to stimulate MDA-MD-231 cell proliferation (Figure 5A), as was the case for MCF-7 cells. Higher doses of the CM extract, however, inhibited ($p < 0.01$) MDA-MD-231 cell proliferation (Figure 5A), and this response was also enhanced by estradiol treatment. Because

MDA-MD-231 cells lack functional ER, the inhibitory interaction between estradiol and CM likely results from toxicity.

Partial purification and characterization of CM from ground corncob. Fractionation and purification of the corncob extract on Spice C18 cartridges demonstrated that the mitogenic activity detected in MCF-7 cell cultures eluted from the cartridge in the 80% methanol fraction (Figure 5B). Lower

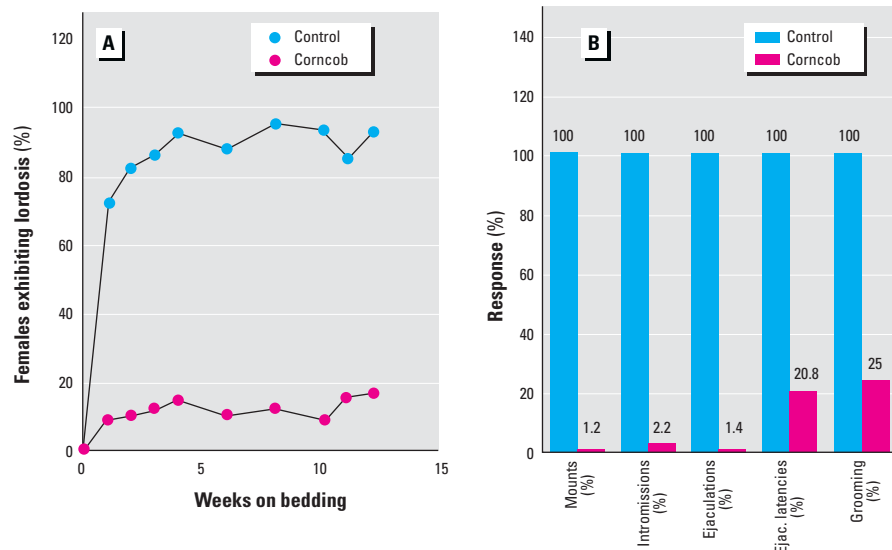


Figure 4. Effects of housing on mating behavior in male and female rats. Ejac. latencies, ejaculation latencies. (A) Adult, ovariectomized rats housed in wire cages (controls) or on ground corncob bedding (corncob) were primed with estrogen plus progesterone, and their sexual behavior (lordosis response) in the presence of proven male rats was scored and quantitated. (B) The sexual performance of adult male proven breeders housed on Sani-chip (control) or ground corncob bedding (corncob) in the presence of sexually receptive ovariectomized rats was evaluated. The numbers of mounts, intromissions, ejaculations, ejaculation latencies in seconds, and grooming frequencies in a 30-min test period with each sexually receptive female were scored.

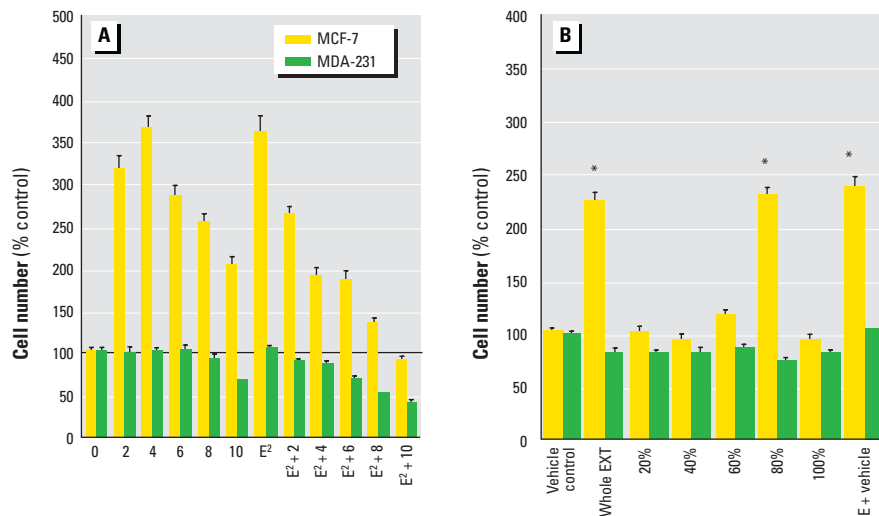


Figure 5. Effects of a corncob bedding extract (EXT) on the proliferation of human breast cancer cells. (A) Aliquots (0–10 μ L) of CM extract were added to exponentially growing cultures of MCF-7 or MDA-MD-231 cells in the presence or absence of 10 nM estradiol for 6 days. (B) The CM extract was fractionated on a Spice C18 reverse-phase cartridge by elution with increasing concentrations (20–100%) of ethanol and aliquots of the fractions were added to the exponentially growing MCF-7 or MDA-MD-231 cells. Treatment effects on cell number were determined 6 days after treatment. * $p < 0.05$.

concentrations of methanol (20–60%) failed to elute the mitogenic agent. The stimulatory response obtained with the 80% methanol fraction was similar to that obtained with an equivalently diluted preparation of the crude extract, suggesting that recovery was quantitative. Surprisingly, both the crude (data not shown) and purified fractions (80% methanol eluate) failed to compete for [³H]estradiol binding to the ER in rat uterine cytosol (Figure 6A) or in uterine nuclear (data not

shown) preparations. The crude or purified extract also failed to significantly modulate ER-mediated gene transcription in HeLa cells transfected with the estrogen-receptor (psvmt:wER) and reporter gene (data not shown) containing an estrogen responsive element (ERE; pERE-ELb-LUC) (36,37). Similarly, treatment with the antiestrogen ICI-182,780 failed to inhibit the stimulation of MCF-7 cell proliferation by the partially purified corn cob bedding extract (Figure 6B).

Under these conditions, this level of antiestrogen reduced the proliferative response of these cells to estradiol by nearly 70%. Thus, although MCF-7, but not MDA-MD-231, cells were stimulated by this mitogen in the corn cob bedding extract, it is unlikely that the ER is directly involved in this response. Similarly, although this partially purified CM competitively inhibited [³H]estradiol binding to nuclear type II sites in rat uterine preparations (Figure 6A), more highly purified preparations were devoid of this activity. Thus, although plant-derived bioflavonoids, their metabolites, and/or analogs may control cellular proliferation via interaction with nuclear type II sites, apparently the CM does not directly regulate breast cancer cell proliferation through this mechanism (11,12). Obviously, downstream effects from either ER or type II binding site interactions cannot be ruled out.

On the basis of the findings with estradiol and ICI-182,780 noted in Figure 6, we reasoned that MDA-MD-231 cells might have failed to proliferate in response to CM (Figure 5A) because these cells are more sensitive to this agent(s). If so, the quantities of CM extract used in the experiments described in Figure 5A might have been at the higher end of the MDA-MD-231 cell dose–response curve where cell growth inhibition would have been expected. This possibility was evaluated in the studies described in Figure 7. When the corn bedding extract was diluted 1- to 1,000-fold before addition to MDA-MD-231 cells, a classic biphasic dose–response curve was obtained. These lower concentrations of the diluted CM

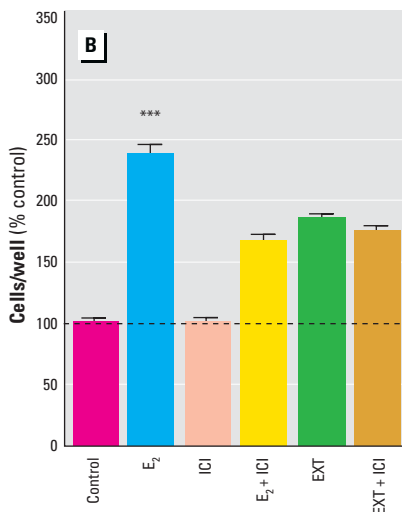
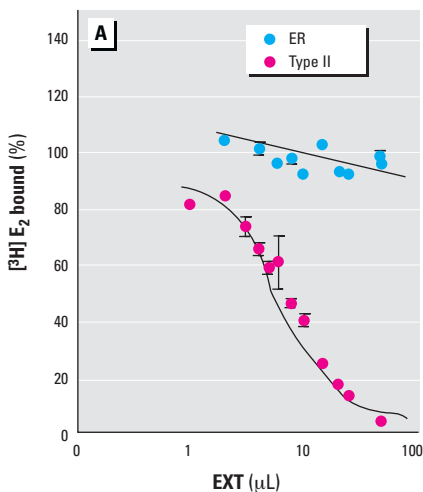


Figure 6. Interaction of corn mitogen extract (EXT) effects with ER and nuclear type II sites in rat uterus and with estrogenic response in MCF-7 human breast cancer cells. (A) Aliquots of the 80% methanol Spice cartridge eluate of CM were diluted (1–50 µL) in ethanol-TE buffer vehicle (control) and added to the cytosol (ER) or nuclear preparations (type II sites) from rat uterus, and [³H]estradiol binding to ER or type II sites was determined. (B) The effects of a pure antiestrogen (ICI-182,780) on the stimulation of MCF-7 cell proliferation by CM preparations were also evaluated.

****p* < 0.001.

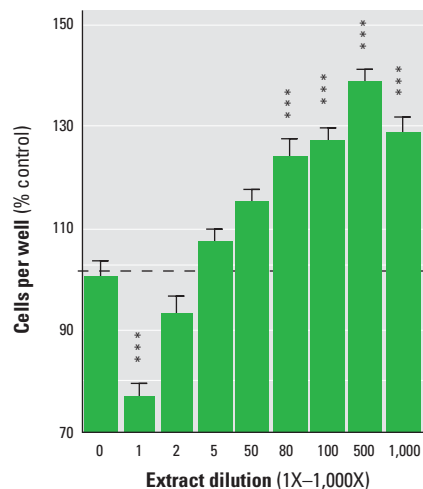


Figure 7. Effects of diluted CM preparations on the MDA-MD-231 cell proliferation. The 80% methanol C18 CM fraction described in Figure 5 was diluted 2- to 1,000-fold in ethanol and 5 µL aliquots of these diluted preparations or 5 µL of 100% glass-distilled ethanol (control) were added to exponentially growing cultures of MDA-MD-231 cells. Cell number was determined 6 days after treatment.

****p* < 0.001.

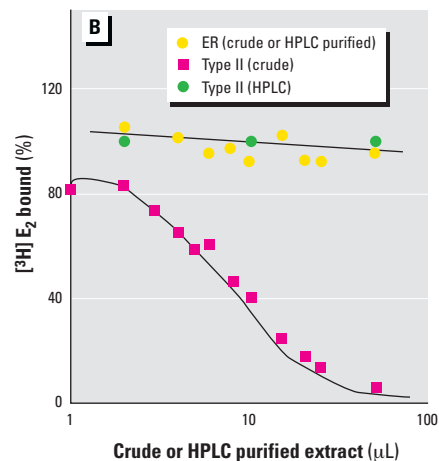
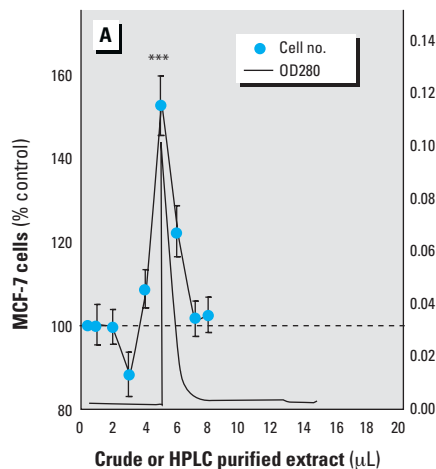


Figure 8. Effects of HPLC-purified CM preparations on MCF-7 human breast cancer cell proliferation and interactions with ER and nuclear type II [³H]estradiol binding sites. An aliquot of the 80% methanol fraction of the CM from a C18 mini-column was injected onto a Waters µBondapak C18 reverse-phase column. (A) Aliquots of fractions (1 min) were reconstituted in 100% ethanol and added to exponentially growing MCF-7 cells for assessment of effects on cellular proliferation. (B) Unlike the partially purified preparations of CM (Figure 6A), the HPLC peak fraction failed to compete for [³H]estradiol binding to ER or nuclear type II [³H]estradiol binding sites.

****p* < 0.001.

extract stimulated cellular proliferation ($p < 0.01$) relative to controls, whereas the higher concentrations (Figure 5A) were inhibitory. Thus, even in rapidly proliferating ER-independent MDA-MD-231 cell populations, CM can stimulate proliferation.

Reverse-phase HPLC of CM preparations. Injection of the fractionated corncob bedding extract (80% methanol eluate from Spice cartridge) onto a Waters μ Bondapak C18 reverse-phase column (Figure 8A) demonstrated that the mitogenic activity (in MCF-7 cells) eluted with a single major peak

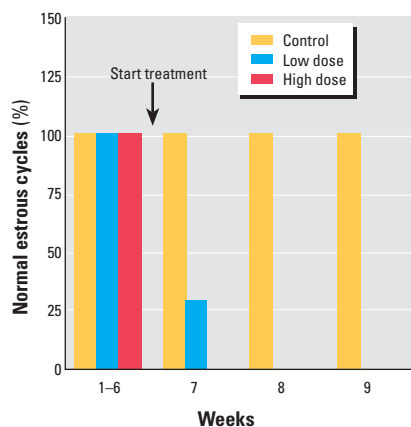
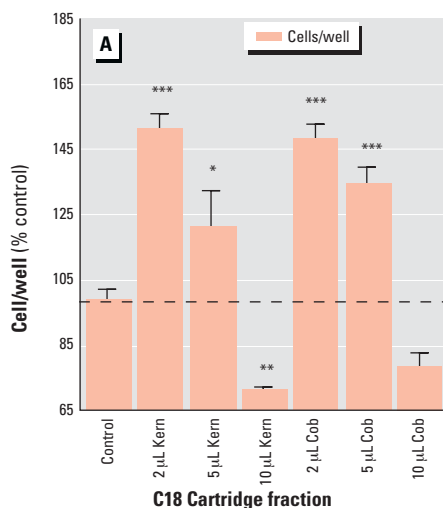


Figure 9. Effects of CM preparations on estrous cycles in adult female rats. Adult female rats housed in wire cages displayed normal 4- to 5-day cycles (weeks 1–6). At the beginning of week 7, the animals (10 per group) were given 2% Tween-80-Tap water vehicle (controls) or a low (50 μ L/400 mL) or high (100 μ L/400 mL) dose of the purified CM extract (80% methanol Spice cartridge eluate). Vaginal smears were collected daily during weeks 7–9.



of UV-absorbing material. Thus the 80% methanol eluate from the Spice cartridge chromatographed as a single major component on HPLC under these experimental conditions. Aliquots of this purified material in the HPLC peak fractions failed to compete for [3 H]estradiol binding to ER or nuclear type II sites (Figure 8B), further ruling out direct modulation of estrogen-dependent pathways by this mitogenic agent. Thus, HPLC purification removed the component capable of competing for type II sites in the crude CM preparations.

Inhibition of ovarian cyclicity by CM preparations. The HPLC analysis revealed that the CM preparations (80% methanol eluate from C18 mini-columns) were suitable for assessment of biologic activity in animal studies. For these experiments, three groups of 10 ovariectomized rats each (controls, low-dose extract, and high-dose extract) were housed in wire cages for 6 weeks, and daily vaginal smears were collected to confirm normal estrous cycles. The animals were then given vehicle (controls), or low- (0.2 mL per 400 mL vehicle) or high-dose (2.0 mL per 400 mL vehicle) of CM (80% methanol eluate from Spice cartridge reconstituted in ethanol) in the drinking water for the next 14 days, and daily vaginal smears were collected. Within 7 days after treatment, 75% of the rats receiving the low dose and 100% of the animals receiving the high dose of bedding extract displayed persistent metestrus smears. By the second week of treatment, 100% of the animals in either group were acyclic (Figure 9). Thus, treatment with the purified bedding extract caused persistent metestrus in these animals, which mimicked the results obtained when the female rats

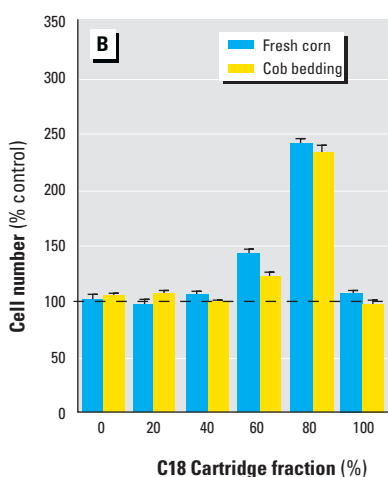


Figure 10. Characterization of mitogenic activity in extracts of fresh corn. (A) Methanol extracts from fresh corn kernels (Kern) or cob purchased in a local supermarket were added to exponentially growing MCF-7 cells and cell number was determined 6 days following treatment. (B) Fractionation of the extract from the corn kernels on Spice C18 cartridges demonstrated that the mitogenic activity eluted with 80% methanol in a manner indistinguishable from that of the ground corncob bedding extract.

* $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$.

were housed on this material (Figure 3). These findings suggest that the mitogenic activity purified from corncob bedding extracts (CM) is likely to be responsible for the disruption of reproductive behavior and function observed in our initial bedding studies.

Extracts from fresh corn kernels or cob also contained this mitogenic activity when assayed in MCF-7 cells (Figure 10A) and the mitogen fractionated on Spice C18 cartridges in a manner indistinguishable (Figure 10B) from that isolated from ground corncob bedding (Figure 5B). A similar mitogenic activity in a corn tortilla extract preparation (80% methanol eluate from C18 mini-columns) was detected after addition to cultured MCF-7 cells (Figure 11), demonstrating that human food products derived from corn contain this mitogenic material. We suspect that corn oil also contains this substance(s) but this has not been investigated.

Effects of bedding material on the proliferation of PC-3 cell human prostatic cancer cells in vitro and in athymic nude mice. CM preparations stimulate the proliferation of estrogen-dependent (MCF-7 cells) and estrogen-independent (MDA-MD-231 cells) human breast cancer cells, suggesting that ER is not involved in the response. Therefore, this mitogenic agent may also stimulate the proliferation of androgen-independent PC-3 human prostatic cancer cells *in vitro* and *in vivo*, as well. We routinely grow PC-3 cells in our laboratory and have used athymic nude mice as hosts for subcutaneous PC-3 cell xenografts to investigate treatment effects on PC-3 cell xenografts *in vivo* (20). Therefore, we used this model to evaluate CM effects on prostatic cancer cell proliferation. These experiments clearly demonstrate that cultured PC-3 cells respond in a dose-dependent manner to the CM preparations, and a typical bell-shaped curve was

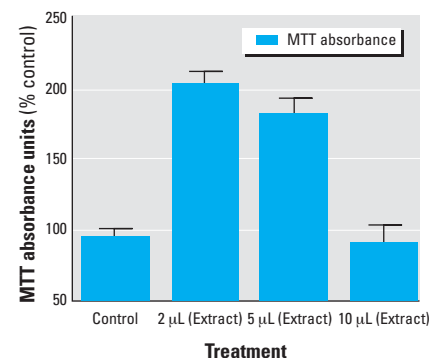


Figure 11. Effects of corn tortilla extracts on the proliferation on MCF-7 human breast cancer cell proliferation. MCF-7 cells were treated with various concentrations (1–10 μ L) of partially purified (80% methanol fraction from C18 mini-column reconstituted in 500 μ L of ethanol) corn tortilla extract or ethanol (controls) for 6 days. Cell proliferation was estimated on the basis of the MTT assay.

obtained (Figure 12A). This finding mirrored results obtained with MCF-7 and MDA-MD-231 cells mentioned above. Furthermore, PC-3 cell xenografts grew more rapidly in nude mice housed on corncob bedding compared to animals housed on pure cellulose (Figure 12B). Thus, the mice apparently adsorbed and/or consumed a component (CM) from corncob bedding that is mitogenic to the PC-3 cell xenografts, enhancing the growth of these xenografts. We believe this factor is CM because preparations of this mitogen directly stimulated PC-3 cells *in vitro* as well. We anticipate that similar results would be obtained with MCF-7 and/or MDA-231 cells in this model system, and this will be evaluated in the future.

Discussion

The studies presented here describe a novel endocrine-disrupting agent purified from ground corncob bedding and fresh corn or tortillas which likely has mitogenic activity in breast and prostatic cancer cells *in vitro* (Figures 5–8, 10–12) and *in vivo* (Figure 12B). We have developed methodology for purifying this mitogenic activity (CM) and the elucidation of the chemical structure of this substance is a major priority of this laboratory. Our present experiments strongly suggest that CM is not a phytoestrogen (37–39), bioflavonoid, or phenolic cell growth regulatory agent such as genistein, luteolin, or methyl-*p*-hydroxyphenyllactate, respectively (20,40). This notion is based on its solubility properties and chromatographic behavior on HPLC. Under the conditions employed in this study, free steroids, bioflavonoids, nonsubstituted fatty acids, and other relatively nonpolar compounds are unlikely to elute. Similarly, the antagonistic effect of CM on uterine growth and estrous cyclicity and particularly on the inability of these preparations to compete for [³H]estradiol binding to ER or nuclear type II sites (Figures 6A and 8B) would suggest that this compound(s) is not free steroid, phenol, bioflavonoid, or bioflavonoid metabolite. It is well documented that phytoestrogens, bioflavonoids, and phenolic metabolites interact with ER and/or nuclear type II [³H]estradiol binding sites (41). Similarly, CM preparations also failed to activate ER-mediated gene transcription in HeLa cells transfected with ER (psvmt:wER) and reporter gene (data not shown) containing an ERE pERE-ELb-LUC, further supporting the concept that CM is regulating cellular proliferation via non-ER-dependent pathways. However, validating these concepts requires further work, particularly in light of recent studies demonstrating that estrogenic induction of mitogen-activated tyrosine protein kinase pathways can occur independent of the ER (42,43). Thus, induction of these pathways may be at least partly

responsible for the response of MCF-7 and MDA-MD-231 breast cancer cells, LNCaP prostate cancer cells, and the uterus to CM in our preparations.

Alternatively, perhaps CM is a progestinogen (antiestrogen) because it induces persistent vaginal metestrus in the rat. It is well known that human breast cancer cells proliferate in response to progesterone (44), a response dependent on the progesterone receptor (PR). However, aliquots of the crude or highly purified corncob bedding extract failed to compete for [³H]progesterone binding to the PR (data not shown) in rat uterine cytosol preparations (45). These findings suggest that CM does not bind to progesterone receptor in a classic manner, although we certainly cannot rule out the possibility that the compound activates progesterone receptor in a ligand-independent manner (17,46). Studies are under way to examine this possibility.

Regardless of its mechanism of action, the present *in vitro* and *in vivo* studies demonstrate that the corncob bedding-derived factor (CM) is an active mitogen in ER-positive and ER-negative breast cancer cells and androgen-independent PC-3 human prostatic cancer cells. Housing rats on ground corncob bedding disrupts male (Figure 4B) and female mating behavior (Figure 4A) and estrous cyclicity (Figures 2 and 3) and stimulates the growth of human prostatic cancer cell xenografts in athymic nude mice (Figure 12). Oral administration of highly purified preparations of the mitogenic agent (CM) to female rats in the drinking water completely disrupted ovarian cyclicity in a dose-dependent manner (Figure 9), and preliminary studies have confirmed a 60% reduction in mating behavior in male rats orally dosed with this CM preparation (data

not shown). Thus, it appears that the mitogenic activity characterized in corncob extracts is responsible for the endocrine-disrupting characteristics of the ground corncob bedding. This will be evaluated directly after the chemical identity of the CM is determined.

Aside from its endocrine-disrupting properties, an agent such as CM must be considered when designing male or female animal reproductive studies. At present, we have not yet identified species other than rats that may be affected by exposure to the corncob bedding material. Rats in our animal facility neither breed nor exhibit reproductive behavior when housed on corncob bedding. In addition, because CM will stimulate mammary and prostatic cancer cell proliferation, it is likely that these unknown agents will affect baseline determinations in reproductive studies in rats and perhaps other species. Perhaps CM will affect mammary and prostate morphology as well. It is clear from our studies with nude mice that exposure to the corncob bedding material accelerates the growth of PC-3 cell xenografts (Figure 12) in these animals. Thus mice are able to adsorb an agent from ground corncob bedding that is indistinguishable from CM at this point. Such agents will obviously affect experimental results in animals housed on this type of bedding material. We have detected CM activity in ground corncob bedding from a number of manufacturers by cell proliferation assays and on HPLC, and this agent(s) is not destroyed by autoclaving. Thus, housing animals on ground corncob bedding, regardless of manufacturer, is likely to influence experimental results in a variety of endocrine-controlled systems. Since ER is not required for response to CM, this compound(s) could be a general mitogen

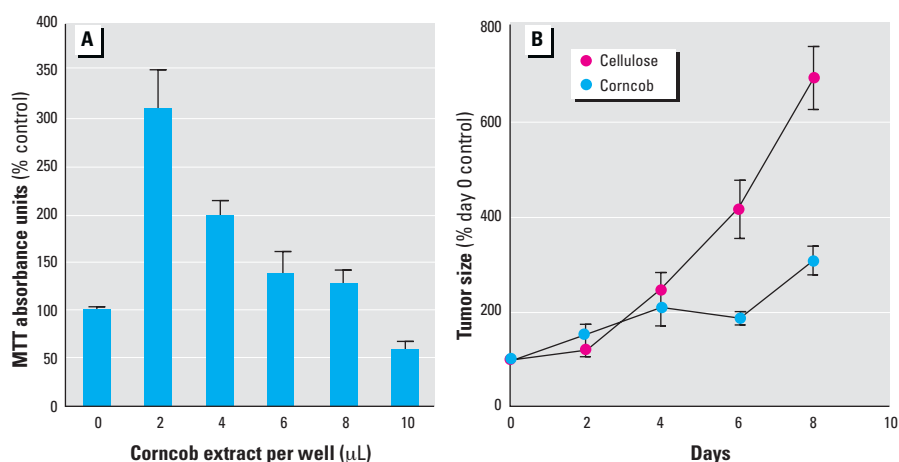


Figure 12. Effects of CM preparations on the proliferation of PC-3 human prostatic cancer cells grown in tissue culture and as xenografts in athymic nude mice. (A) Exponentially growing PC-3 cells were treated with the indicated quantities of CM (80% methanol fraction for C18 cartridges). Cell number was determined by the MTT assay 6 days after treatment. (B) The effects of housing conditions (pure cellulose or corncob bedding) on the growth of subcutaneous PC-3 human prostatic cancer cell xenografts in athymic nude mice (6–10 mice per group). Tumor size (length × width) was monitored throughout the study and expressed as a percentage of the day 0 control value.

affecting all cell types, and this will be evaluated in the future.

In summary, although the potential effects of CM on human populations remains to be resolved, the studies described in this article show that in addition to ground corncob bedding, fresh corn on the cob (kernels and cob) and corn tortillas contain CM. The CM activity from these foods stimulates breast and prostatic cancer cellular proliferation and will likely disrupt endocrine function and behavior as well. CM does not bind to the ER or activate ER-dependent gene transcription in HeLa cells transfected with ER and ERE-luciferase reporter gene constructs (data not shown), and its effects on MCF-7 human breast cancer cell proliferation are not blocked by a pure antiestrogen (ICI-182,780). Thus, CM can stimulate estrogen-dependent and estrogen-independent breast and prostatic cancer cell proliferation at very low doses. These results suggest that CM may be able to stimulate the proliferation of a wide variety of normal and abnormal cells in reproductive tissues and perhaps nonreproductive tissues as well. Determination of the mechanism of action of CM and its metabolic fate in laboratory animals and man is crucial for estimating its impact on human health and development and identifying other natural sources for this type of environmentally derived endocrine-disrupting/mitogenic agent. It is well known that ground or milled corncob is also used for metal finishing and blast cleaning, for litter and bedding for pets and small animals (as shown here), as an absorbent for soaking up aqueous spills, and as a pesticide carrier for fire ant and grub control, feed additives, animal health products, and feed. Academic institutions routinely use ground corncob bedding to house experimental animals used in scientific research. Thus, CM present in corn products may complicate experimental results in animal studies, and human exposure to this endocrine-disrupting agent is likely. Although it is well established that consumption of high-fat diets (23–24% corn oil) increases the incidence, growth, and metastases of mammary, liver, and colon cancer in a variety of experimental systems (47–49), the question of whether acute or sustained exposure to CM and/or related compounds, alone or in combination, represents a significant health problem remains to be resolved.

REFERENCES AND NOTES

- Brown N, Lamartiniere C. Xenoestrogens alter mammary gland differentiation and cell proliferation in the rat. *Environ Health Perspect* 103:708–713 (1995).
- Whitten PL, Naftolin F. Effects of phytoestrogen diet on estrogen-dependent reproductive processes in immature female rats. *Steroids* 57:56–61 (1992).
- Baird D, Umbach D, Lansdell L, Hughes C, Setchell D, Weinberg C, Haney A, Wilcox A, McLachlan J. Dietary intervention study to assess estrogenicity of dietary soy among postmenopausal women. *J Clin Endocrinol Metab* 80:1685–1690 (1995).
- Cassidy A, Bingham S, Setchell K. Biological effects of isoflavones present in soy in premenopausal women: implications for the treatment of breast cancer. *J Clin Nutr* 60:333–340 (1996).
- Cassidy A. Physiological effects of phyto-oestrogens in relation to cancer and other human health risks. *Proc Nutr Soc* 55:399–417 (1996).
- Noble RL, Hochachka CB, King D. Spontaneous and estrogen-produced tumors in Nb rats and their behavior after transplantation. *Cancer Res* 35:766–780 (1975).
- King C. Tamoxifen and the induction of cancer. *Carcinogenesis* 16:1449–1454 (1995).
- Martin PM, Horwitz KB, Ryan DS, McGuire WL. Phytoestrogen interactions with estrogen receptors in human breast cancer cells. *Endocrinology* 103:1860–1867 (1978).
- Port C, Kaltenback J. The effect of corncob bedding on reproductivity and leucine incorporation in mice. *Lab Anim Care* 19:46–49 (1969).
- Pathre SV, Mirocha CJ. Mycotoxins as estrogens. In: *Estrogens in the Environment* (McLachlan JA, ed). New York:Elsevier/North Holland, 1980:265–278.
- Markaverich BM, Roberts RR, Alejandro MA, Johnson GA, Middlelitch BS, Clark JH. Bioflavonoid interactions with rat uterine type II binding sites and cell growth inhibition. *J Steroid Biochem* 29:71–78 (1988).
- Markaverich BM, Gregory RR, Alejandro MA, Clark JH, Johnson GA, Middlelitch BS. Methyl p-hydroxyphenyllactate: an inhibitor of cell proliferation and an endogenous ligand for nuclear type II binding sites. *J Biol Chem* 263:7203–7210 (1988).
- NIH. *Guide for the Care and Use of Laboratory Animals*. Bethesda, MD:National Institutes of Health, 1996.
- Markaverich BM, Roberts RR, Alejandro MA, Clark JH. The effect of low dose continuous exposure to estradiol on the estrogen receptor (type I) and nuclear type II sites. *Endocrinology* 114:814–820 (1984).
- Zarrow M, Yochim J, McCarthy J, eds. *Experimental Endocrinology: A Sourcebook of Basic Techniques*. New York:Academic Press, 1964.
- Hull E, Bazett TJ, Warner RK, Eaton R, Thompson J. Dopamine receptors in the ventral tegmental area modulate male sexual behavior in rats. *Brain Res* 512:1–6 (1990).
- Mani S, Allen J, Clark J, Blaustein J, O'Malley B. Convergent pathways for steroid hormone and neurotransmitter-induced rat sexual behavior. *Science* 256:1246–1249 (1994).
- Mani S, Blaustein D, Allen J, Law S, O'Malley B, Clark J. Inhibition of rat sexual behavior by antisense oligonucleotides to the progesterone receptor. *Endocrinology* 135:1409–1414 (1994).
- Markaverich BM, Roberts RR, Finney RW, Clark JH. Preliminary characterization of an endogenous inhibitor of [³H]estradiol binding in rat uterine nuclei. *J Biol Chem* 258:11663–11671 (1983).
- Markaverich BM, Alejandro MA. Type II [³H]estradiol binding site antagonists: inhibition of normal and malignant prostate cell growth and proliferation. *Cancer Res* 12:1127–1136 (1998).
- Vignon F, Terqui B, Westley B, Deroq D, Rochefort. Effects of plasma estrogen sulfates in mammary cancer cells. *Endocrinology* 106:1079–1086 (1980).
- Markaverich BM, Clark JH. Two binding sites for estradiol in rat uterine nuclei: relationship to uterotrophic response. *Endocrinology* 105:1458–1462 (1979).
- Markaverich BM, Varma M, Densmore CL, Schauweker TH, Gregory RR. Nuclear type II [³H]estradiol binding sites in MCF-7 human breast cancer cells: binding interactions with 2,6-bis[3,4-dihydroxyphenyl]-methylene]-cyclohexanone esters and inhibition of cell proliferation. *Int J Oncol* 4:1291–1300 (1994).
- Markaverich BM, Upchurch S, Williams M, Clark JH. Heterogeneity of nuclear estrogen-binding sites in the rat uterus: a simple method for the quantitation of type I and type II sites by [³H]estradiol exchange. *Endocrinology* 109:62–69 (1981).
- Tremblay G, Tremblay A, Copeland N, Giquere V. Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor α . *Mol Endocrinol* 11:353–365 (1997).
- Kuiper GJGM, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson J-A. Cloning of a novel estrogen receptor expressed in rat prostate and ovary. *Proc Natl Acad Sci USA* 93:5925–5930 (1996).
- Markaverich B, Gregory R, Alejandro M, Kittrell F, Medina D, Clark J, Varma M, Varma R. Methyl p-hydroxyphenyllactate and nuclear type II binding sites in malignant cells: metabolic fate and mammary tumor growth. *Cancer Res* 50:1470–1478 (1990).
- Faircloth G, Stewart D, Clement J. A simple screening procedure for the quantitative measurement of cytotoxicity to resting primary lymphocyte cultures. *J Tissue Cult Methods* 11:201–205 (1990).
- Markaverich BM, Upchurch S, Glasser SR, McCormack SA, Clark JH. Estrogen stimulation of uterine growth: mechanism of steroidal and non-steroidal estrogen antagonists. In: *Non-Steroidal Anti-Oestrogens* (Jordan RLSaVC, ed). Australia:Academic Press, 1981:113–142.
- Whitten PL, Russel E, Naftolin F. Effects of a normal, human-concentration, phytoestrogen diet on rat uterine growth. *Steroids* 57:98–106 (1992).
- Whitten PL, Naftolin F. Xenoestrogens and neuroendocrine development. In: *Prenatal Exposure to Toxicants: Developmental Consequences* (Needleman HL, Bellinger D, eds). Baltimore, MD:Johns Hopkins University Press, 1996:269–293.
- Coopman P, Garcia M, Brunner N, Deroq D, Clarke R, Rochefort H. Anti-proliferative and anti-estrogenic effects of ICI 164,384 and ICI 182,780 in 4-OH tamoxifen-resistant human breast cancer cells. *Int J Cancer* 56:295–300 (1994).
- Maier PA. Nuclear translocation of fibroblast growth factor (FGF) receptors in response to FGF-2. *J Cell Biol* 134:529–536 (1996).
- Miller MA, Katzenellenbogen BS. Characterization and quantitation of antiestrogen binding sites in estrogen receptor-positive and -negative human breast cancer cell lines. *Cancer Res* 43:3094–3100 (1983).
- Weigel R, DeConinck E. Transcriptional control of estrogen receptor in estrogen receptor-negative breast carcinoma. *Cancer Res* 53:3472–3474 (1993).
- Smith D, Toft D. Steroid hormone receptors and their associated proteins. *Mol Endocrinol* 7:4–11 (1993).
- Mäkelä S, Santti R, Salo L, McLachlan JA. Phytoestrogens are partial estrogen agonists in the adult male mouse. *Environ Health Perspect* 103(suppl 7):123–127 (1995).
- Kamboj S, Shangary S, Singh J, Kamboj K, Sandhu R. New lymphocyte stimulating monocot lectins from family Araceae. *Immunol Invest* 24:845–855 (1995).
- Thompson L. Antioxidants and hormone-mediated health benefits of whole grains. *Crit Rev Food Sci Nutr* 43:473–497 (1994).
- Markaverich BM, Alejandro MA. Bioflavonoids, type II [³H]estradiol binding sites and prostatic cancer cell proliferation. *Int J Oncol* 11:1311–1319 (1998).
- Patisaul H, Whitten P. *Dietary Phytoestrogens*. Boca Raton, FL: CRC Press, 1999.
- Filardo E, Quinn J, Bland K, Frackelton A Jr. Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, and occurs via trans-activation of the epidermal growth factor receptor through release of HB-EGF. *Mol Endocrinol* 14:1649–1660 (2000).
- Migliaccio A, Castorie G, Di Domenico M, Lombardi M, Barone M, Ametrano D, Zannini M, Abbondanza C, Aurichio F. Steroid-induced androgen receptor-estradiol receptor α -Src complex triggers prostate cancer cell proliferation. *EMBO J* 19:5406–5417 (2000).
- Dran G, Luthy I, Molinolo A, Montecchia F, Charreau E, Pasqualinin C, Lanari C. Effect of medroxyprogesterone acetate (MPA) and serum factors on cell proliferation in primary cultures of an MPA-induced mammary adenocarcinoma. *Breast Cancer Res Treat* 32:173–186 (1995).
- Walters MR, Clark JH. *Progesterone Receptors in Normal and Neoplastic Tissues*. New York:Raven Press, 1979.
- Power RF, Mani SK, Codina J, Conneely OM, O'Malley BW. Dopaminergic and ligand independent activation of steroid hormone receptors. *Science* 254:1636–1639 (1991).
- Hilakivi-Clarke L, Clarke R, Onojai I, Raygada M, Cho E, Lippman M. A maternal diet high in n-6 polyunsaturated fats alters mammary gland development, puberty onset, and breast cancer risk among female rat offspring. *Proc Natl Acad Sci USA* 94:9372–9377 (1997).
- Singh J, Hamid R, Reddy B. Dietary fat and colon cancer: modulation of cyclooxygenase-2 by types and amount of dietary fat during postnatalization stage of colon carcinogenesis. *Cancer Res* 57:3465–3470 (1997).
- Weisburger J, Rivenson A, Hard G, Nagao M, Sugimura T. Role of fat and calcium in cancer causation by food mutagens, heterocyclic amines. *Proc Soc Exp Biol Med* 205:347–352 (1994).