Inhibition of Gap Junctional Intercellular Communication in Normal Human Breast Epithelial Cells after Treatment with Pesticides, PCBs, and PBBs, Alone or in Mixtures

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Chemical pollutants in the Great Lakes have found their way through the food chain into humans because of their environmental persistence and lipophilicity. Some epidemiological studies have claimed an association between metabolites of 2,2-bis(p-chlorophenyl)-1,1,1-trichloroethane (DDT), polychlorinated biphenyls (PCBs), and polybrominated biphenyls (PBBs) and breast cancer, but others have reported no such association. We examined various halogenated hydrocarbons for their capacity to inhibit gap junctional intercellular communication (GJIC) in normal human breast epithelial cells (HBEC) when given as single compounds or as mixtures. The scrape-loading/dye transfer and fluorescent redistribution after photobleaching techniques were used to measure GJIC, immunostaining and Western and Northern analyses were performed on connexin 43 (Cx43) gap junction protein and message to determine how halogenated hydrocarbons might affect GJIC.

DDT, dieldrin, and toxaphene inhibited GJIC in a dose-responsive manner after 90 min treatments. Dieldrin suppressed GJIC within 30 min with no recovery after 24 hr. Inhibition of GJIC by DDT and toxaphene was partially restored after 12 hr and fully restored after 24 hr. Several PCB and PBB congeners inhibited GJIC in a dose-responsive and time-dependent manner, but GJIC was almost restored to control values 24 hr after exposure. The highest concentrations of the individual chemicals that did not inhibit GJIC was determined, and mixtures containing two of these chemicals were tested for their ability to inhibit GJIC. Significant inhibition of GJIC was observed when cells were treated with a mixture of DDT and 2,4,5-hexachlorobiphenyl (2,4,5- HCB), dieldrin and 2,4,5-HCB, or dieldrin and 2,4,5-hexabromobiphenyl (2,4,5-HBB). These results indicate that halogenated hydrocarbons, alone or in specific combinations, can alter GJIC at the post-translational level. These results are consistent with the hypothesis that DDT, dieldrin, toxaphene, 2,3,4-HCB, 2,4,5-HCB, and 2,4,5-HBB could have tumor-promoting potential in human breast tissue. Key words chemical mixtures, DDT, dieldrin, gap junctional intercellular communication, normal human breast epithelial cells, PCBs, PBBs, toxaphene. Environment Health Perspectives 104:192–200 (1996).

Breast cancer is one of the most commonly diagnosed cancers in women (1,2). Breast cancer incidence has been increasing steadily in most highly developed countries (3). This trend has been attributed to risk factors such as more women smoking and increased oral contraceptive use, as well as early menarche, late menopause, family history, “delayed” childbearing, and potential exposure to various environmental chemicals (4–7). There is growing concern that a large number of human-made chemicals that have been released into the environment and that have been found to persist and travel through the food chain, can cause cancers in laboratory rodents (8–10). Among these chemicals, pesticides, herbicides, polychlorinated biphenyls (PCBs), and polybrominated biphenyls (PBB) have been found in both aquatic and soil matrices and in the food chain associated with these environments (8–11).

Many of these chemicals have accumulated in animals and humans through several routes of exposure. These accumulated lipophilic halogenated hydrocarbons have been speculated to play a causative role in various human diseases (12–18). PCBs are embryotoxic in mink, neurotoxic during development in animals, and can alter uterine progesterone and progesterone receptors (17,19). Although some bioassay data on rodents suggest that many of these pesticides and chemicals, such as the PCBs and PBBs, are carcinogens, in vitro and in vivo initiation–promotion bioassays suggest that most of these chemicals are acting as tumor promoters via nongenotoxic or epigenetic mechanisms (20).

Due to their lipophilic nature, these chemicals readily accumulate in animal and human tissues. Human breast tissue appears to be a site for some of the accumulation of these chemicals. Because of the estrogenic activity of some of these chemicals, and the bioaccumulation in human breast tissue, epidemiological studies have been performed to test if DDT metabolites and PCBs might be etiological agents of human breast cancers. Studies to date have not resolved the hypothesis that this class of pollutant, “estrogenlike” chemicals, have an influence on human breast cancer (21–34).

Because carcinogenesis is a multistep, multimechanism process, consisting of the operational steps of initiation, promotion, and progression (35), and because both genotoxic and epigenetic mechanisms play a role in this complex process, it is important to consider these concepts in the design and interpretation of both animal bioassays and epidemiological studies of cancer. One of the facts related to the concern that these chemicals be assessed for their carcinogenic potential on human breast tissue is that data showing that most of these chemicals (DDT, PCB, PBB, dieldrin, etc.) are not genotoxic in rodent cells but are rather effective tumor promoters of liver cancers in rodents (36–38).

In addition, most tumor promoters (e.g., phorbol esters, phenobarbital, saccharin, TCDD, DDT, dieldrin, toxaphene, PCBs, PBBs) have the ability to inhibit, in a reversible manner, gap junctional intercellular communication (GJIC) in various cells, in vitro, at noncytotoxic but at concentrations sufficient (e.g., threshold concentrations) to inhibit GJIC (37–41). Inhibition of the communication of ions and small molecular weight molecules through these transmembrane protein channels between contiguous cells has been postulated to release initiated cells from the suppressing effects of signals passing from surrounding normal cells (42,43). Therefore, we designed studies to determine if several of the environmental chemicals found in the Great Lakes are able to inhibit GJIC in normal human breast epithelial cells, either alone or in specific combination with other
chemicals. Results clearly indicate that these chemicals could block GJIC in these cells, and the concentrations needed to do so, in vivo, were lower when these chemicals were given in certain mixtures.

Materials and Methods

Cell cultures. Normal human breast epithelial cells (HBEC) were obtained from reduction mammoplasty tissue specimens from seven patients (44). Tissue specimens were minced into small pieces with scalpels, then digested in collagenase-type IA (Sigma, St. Louis, Missouri) solution (1 g tissue/10 mg of collagenase in 10 ml medium) at 37°C in a waterbath for 16–18 hr. The digested tissue solution was then centrifuged to remove the collagenase solution.

Cellular pellets were washed once with MSU-1 medium (44), then resuspended in the MSU-1 medium supplemented with 5% fetal bovine serum (FBS; Gibco-BRL, Gaithersburg, Maryland). The cells were plated into two flasks (150 cm²). After 2 hr incubation, the cells (or aggregates) which remained in suspension were transferred to 4–6 flasks (75 cm²) to reduce the number of attached fibroblasts. After overnight incubation, the medium was changed to the FBS-free MSU-1 medium. The MSU-1 medium was changed once every 2 days for 1 week. The cells were cultured in D-medium:MCDB (1:1; Sigma) without FBS (MSU-1). This modified Eagle's minimal essential medium was supplemented with recombinant human epidermal growth factor (1 ng/ml), insulin (10 μg/ml), hydrocortisone (1 ng/ml), 17β-estradiol (10 nM), and 0.4% bovine pituitary extract. All cells were grown at low passage at 37°C and 5% CO₂ in a humidified incubator. The cells were passaged by trypsinization.

The first passage of HBEC, recovered from liquid nitrogen, was plated in MSU-1 medium supplemented 5% FBS. After 4 hr incubation, the cells which remained in suspension were transferred to new plates (100 mm²) in FBS-free MSU-1 medium supplemented with 0.4% bovine pituitary extracts (Pel-Freez, Roger, Arkansas), where they gave rise to a second morphological type of cells (44). We used this second morphological type of cell (2–3 passages) for the experiments described here.

Chemicals. PCBs, PBBs, 4,4'-DDT, dieldrin, and toxaphene were obtained from Chem Service Co. (West Chester, Pennsylvania). Additional reagents were purchased from Sigma.

Cytotoxicity test. Cells (1 x 10⁴) were plated in triplicate in 96-well, flat-bottomed microtiter plates. Twenty-four hours later, the medium was changed and fresh medium with the test chemicals was added. Twenty-four hours after treatment, the medium was removed, and cells were washed with warm phosphate-buffered saline (PBS) three times and then 200 ml of neutral red medium (50 mg/ml in growth medium) was added to each well. Plates were incubated at 37°C for 2 hr, and then cells were solubilized with 1% acetic acid in 50% ethanol for 20 min. We determined absorbance at 540 nm and 630 nm for each well using 96-well plate reader (Bio-Tek Instruments, Winooski, Vermont).

HBEC type II cells were cultured in 35-mm dishes to 90–100% confluence, then treated with PCBs, PBBs, DDT, toxaphene, and dieldrin. We dissolved 2,2',3,3',4,4'–HCB, 2,2',4,4',5,5'–HBB, 2,2',4,4',5,5'–HCB in dimethylsulfoxide and 3,3',4,4',5,5'–HCB, 3,3',4,4',5,5'–HBB in dimethylsulfoxide:acetone (1:1). Dieldrin and 4,4'-DDT were dissolved in ethanol; toxaphene was dissolved in acetone. The maximum volume of the chemicals applied to the cells was 5 μl/ml. Control cells were treated with each solvent (5 μl/ml).

Gaps junctional intercellular communication assays. We measured gap junction-mediated intercellular communication using the dye transfer technique (45). The cultures were rinsed with PBS, and then 2.0

| Chemical          | Dose (μM) | No. of cells | Rate of dye transfer/min | % of control |
|-------------------|-----------|--------------|--------------------------|--------------|
| 2,4,5-HCB         | 27        | 4.73 ± 1.84  | 100                      |              |
|                   | 27        | 4.40 ± 1.84  | 93                       |              |
|                   | 28        | 3.57 ± 1.58  | 75                       |              |
|                   | 56        | 4.29 ± 1.82  | 91                       |              |
|                   | 70        | 1.56 ± 1.00  | 33                       |              |
| 3,4,5-HCB         | 27        | 4.73 ± 1.84  | 100                      |              |
|                   | 27        | 5.37 ± 2.23  | 113                      |              |
|                   | 28        | 4.46 ± 1.42  | 94                       |              |
|                   | 56        | 5.95 ± 2.60  | 126                      |              |
| 2,4,5-HBB         | 37        | 5.56 ± 1.04  | 100                      |              |
|                   | 40        | 4.92 ± 0.63  | 89                       |              |
|                   | 8         | 4.84 ± 1.85  | 87                       |              |
|                   | 16        | 4.16 ± 1.23* | 80                       |              |
|                   | 32        | 2.01 ± 1.24* | 36                       |              |
|                   | 64        | 1.88 ± 0.82* | 30                       |              |
| 3,4,5-HBB         | 37        | 5.56 ± 2.04  | 100                      |              |
|                   | 20        | 5.74 ± 1.56  | 103                      |              |
|                   | 40        | 8.02 ± 2.07  | 144                      |              |
|                   | 80        | 5.43 ± 1.92  | 98                       |              |
|                   | 160       | 6.81 ± 2.03  | 120                      |              |
| 2,3,4-HCB         | 37        | 5.56 ± 2.04  | 100                      |              |
|                   | 9         | 5.92 ± 2.30  | 106                      |              |
|                   | 18        | 4.75 ± 2.06  | 85                       |              |
|                   | 35        | 4.52 ± 2.43  | 81                       |              |
|                   | 70        | 0.14 ± 0.07* | 3                        |              |
| DDT               | 24        | 4.51 ± 0.90  | 100                      |              |
|                   | 6         | 4.43 ± 0.54  | 98                       |              |
|                   | 13        | 3.89 ± 1.00  | 82                       |              |
|                   | 25        | 0.78 ± 0.35  | 17                       |              |
|                   | 50        | 0.31 ± 0.07* | 7                        |              |
|                   | 100       | 0.33 ± 0.17* | 7                        |              |
| Dieldrin          | 24        | 4.51 ± 0.80  | 100                      |              |
|                   | 3         | 4.04 ± 0.24  | 90                       |              |
|                   | 6         | 3.27 ± 1.87  | 73                       |              |
|                   | 13        | 1.53 ± 0.91* | 34                       |              |
|                   | 25        | 1.21 ± 1.36* | 27                       |              |
|                   | 50        | 0.80 ± 0.39* | 18                       |              |
| Toxaphene         | 20        | 6.02 ± 1.61  | 100                      |              |
|                   | 1.25      | 5.31 ± 1.46  | 88                       |              |
|                   | 2.50      | 5.21 ± 2.20  | 87                       |              |
|                   | 5.00      | 2.10 ± 0.85* | 35                       |              |
|                   | 10.00     | 0.69 ± 0.42* | 11                       |              |

*Values represent mean ± SD of the recovery rate of fluorescence dye per minute obtained from fluorescence redistribution after photobleaching assay for 9 min.
*Cytotoxicity was observed.
*Significantly different from vehicle control (p<0.05).
ml PBS containing 0.05% Lucifer yellow DH-rhodamine dextran (Sigma) was added to the petri dishes. The dye was loaded into the primary recipient cells by making six scrape lines on the monolayer with a sharp surgical blade. After 3 min of loading the cells with the dye, the solution was removed and the cells were washed with PBS. The plates were examined under Nikon epifluorescence inverted microscope with an appropriate filter.

To measure gap junctional communication using the fluorescence redistribution after photobleaching (FRAP) assay, after 24 hr of growth, the cells were washed with PBS containing calcium (0.9 mM) and magnesium (0.5 mM; PBS/Ca/Mg) and stained with 6-carboxyfluorescein diacetate for 9 min (46). The plates were then washed several times with PBS/Ca/Mg. The FRAP assay was performed using the Ultima image analyzer (Meridian Co., Okemos, Michigan). Individual cells were photobleached with a 488 nm laser and recovery of fluorescence intensity monitored at 3-min intervals for 9 min. Fluorescence intensity was corrected for photobleaching, which occurs during scanning of the control areas.

Immunofluorescent staining of gap junctions. We immunostained connexin 43 (Cx43)-containing gap junctions in HBEC using a rabbit polyclonal antibody against Cx43. After treatment, the cells were fixed in cold 50% methanol and 50% acetone for 3 min and rehydrated with PBS; subsequently, nonspecific sites were blocked with 10% normal goat serum (NGS) in PBS for 30 min at room temperature. The cells were incubated with anti-Cx43 diluted 1:100 in PBS for overnight at 4°C, washed three to four times with PBS, and then incubated with rhodamine-conjugated goat anti-rabbit IgG Fab2 fraction (Jackson Immuno Research Laboratories, West Grove, Pennsylvania) diluted 1:50 in PBS for 30 min at room temperature. The cells were washed extensively with PBS, and coverslips were mounted in Poly-

Figure 1. Dose-responsive inhibition of gap junctional intercellular communication in normal human breast epithelial cells treated with different concentrations of DDT using the scrape-loading dye transfer assay. (A) Control; (B) 6 μM; (C) 13 μM; (D) 25 μM; (E) 50 μM; (F) control (phase-contrast).

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amid gels and transferred to PVDF membranes at 20 V for 16 hr. We detected Cx43 using anti-connexin 43-specific monoclonal antibody (Zymed, Inc., San Francisco, California), followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody and detected with the ECL chemiluminescent detection reagent (Amersham Co., Arlington Heights, Illinois). The membranes were exposed to X-ray film for 30 sec.

**Alkaline phosphatase treatment.** Samples (20 μg) of the alkali-resistant membrane protein fractions were diluted twofold with reaction buffer (50 mM Tris, pH 8.5; 2 mM PMSF; 0.1% β-mercaptoethanol; 8 mM MgCl₂) and incubated with and without 10 U of calf intestine alkaline phosphatase (10,000 U/ml; New England Biolob, Beverly, Massachusetts) for 2 hr at 37°C. The proteins in the reactions were separated by SDS-PAGE and then blotted to PVDF membranes for Western blot analysis.

**Northern blotting for Cx43 mRNA.** We isolated total RNA from HBEC by guanidium thiocyanate extraction (48). The isolated RNA was separated by electrophoresis on 1.2% agarose-6.7% formaldehyde gels, transferred onto a nylon membrane (Hybond N⁺; Amersham Co., Arlington Heights, Illinois). The membranes were dried for 2 hr under vacuum at 80°C, prehybridized for 1 hr at 63°C, and then hybridized with a digoxigenin-dUTP-labeled Cx43 cDNA using a random-primed DNA labeling kit (Boehringer Mannheim, Indianapolis, Indiana). The hybridized RNA bands were detected on film by exposure to light generated by chemiluminescent reaction using the anti-digoxigenin-AP antibody and Lumi-phos 530.

**Statistics.** We analyzed data by ANOVA (analysis of variance). The significance level (0.05) is compared with the calculated F-value. If there was a difference among groups, data were further analyzed to determine where the difference was by post-hoc Dunnett's t-test at a significance level of p<0.05.

### Results

**Dose Selection Using Cytotoxicity Assay**

To determine the cytotoxicity of these chemicals on HBEC, we examined the uptake of neutralred dye in viable cells using a microplate reader (OD 540 nm and 630 nm). We observed cytotoxicity after a 24-hr exposure to the following mixture of chemicals above these dose levels: 2,2',4,4',5,5'-HCB, 70 μM; 3,3',4,4',5,5'-HCB, 56 μM; for 2,2',4,4',5,5'-HBB, 64 μM; 3,3',4,4',5,5'-HBB, 160 μM; 2,2',3,3',4,4'-HBB, 70 μM; DDT, 100 μM; dieldrin, 100 μM; and toxaphene, 10 μg/ml (mixture of compounds). Because it is important to measure a chemical's effect on GJIC at nontoxic doses, we determined the maximal nontoxic doses. On the basis of these dose levels, we selected doses to be used at a twofold dilution, up to the maximum nontoxic levels.

**Effects on GJIC**

Among PCBs and PBBS, 2,2',4,4',5,5'-HCB (0–70 μM) and 2,2',4,4',5,5'-HBB (0–64 μM) inhibited GJIC in HBEC in a dose-dependent manner after a 90-min treatment, whereas the coplanar structures...
of these chemicals, 3,3',4,4',5,5'-HCB (0-56 μM) and 3,3',4,4',5,5'-HBB (0-160 μM) did not inhibit GJIC. 2,2',3,3',4,4'-HCB (0-70 μM) also slightly inhibited GJIC in a dose-dependent manner (Table 1). DDT (0-100 μM), dieldrin (0-50 μM), and toxaphene (0-10 μg/ml) inhibited GJIC in a dose-dependent manner after a 90-min treatment, as assayed by fluorescence dye transfer (Fig. 1).

Dose levels of 70 μM 2,2',4,4',5,5'-HCB, 70 μM 2,2',3,3',4,4'-HCB, 64 μM 2,2',4,4',5,5'-HBB, 25 μM-100 μM DDT, 12.5-50 μM dieldrin, and 5-10 μg/ml toxaphene significantly inhibited GJIC in HBEC (p<0.05).

2,2',4,4',5,5'-HCB (28 μM), 3,3',4,4',5,5'-HBB (56 μM), 2,2',3,3',4,4'-HCB (56 μM), 2,2',4,4',5,5'-HBB (32 μM), and 3,3',4,4',5,5'-HBB (16 μM) inhibited GJIC in a dose-dependent manner as measured by the fluorescence dye transfer assay (Table 2). Significant inhibition of GJIC was observed in HBEC treated with 2,2',4,4',5,5'-HCB 6-12 hr after treatment, 3,3',4,4',5,5'-HBB 24 hr after treatment, 2,2',3,3',4,4'-HCB 3-6 hr after treatment, 2,2',4,4',5,5'-HBB 3-12 hr after treatment, and 3,3',4,4',5,5'-HBB 3-12 hr after treatment (p<0.05). After 24-hr exposure to these chemicals, the rates of dye recovery were restored to 70-80% of control levels (Table 2).

The time-dependent manner by which DDT (50 μM), dieldrin (25 μM), and toxaphene (10 μg/ml) inhibited GJIC in HBEC is shown in Table 3. Dieldrin and toxaphene inhibited GJIC by 70-80% after 3 hr and 30 min of exposure in HBEC, respectively. Recovery of GJIC was not observed within 24 hr. However, although DDT inhibited GJIC by 50% after 12-hr exposure, recovery of GJIC to 75% of control levels was seen within 24 hr.

DDT (6 μM), dieldrin (3 μM), 2,2',4,4',5,5'-HCB (14 μM), and 2,2',4,4',5,5'-HBB (8 μM) did not inhibit GJIC after a 90-min treatment in HBEC at the lower dose levels used, compared with controls (Fig. 3). However, among these chemicals, the mixtures of DDT (6 μM) and 2,2',4,4',5,5'-HCB (14 μM), as well as dieldrin (3 μM) and 2,2',4,4',5,5'-HCB (14 μM), significantly inhibited GJIC (p<0.05; Figs. 2D-F, 3).

Connexin 43 Proteins
Membrane-localized gap junctional plaques were readily apparent in control cells (Fig. 4A). Cells treated for 90 min with dieldrin (25 μM; Fig. 4C), 2,2',4,4',5,5'-HBB (28 μM; Fig. 4D), DDT (50 μM; data not shown) in membrane plaques of gap junction were missing from the cell membranes. This change was accompanied by numerous cytoplasmic spots (Fig. 4D), suggestive of gap-junction internalization. Furthermore, fewer plaques in cells were detected after treatment with the mixtures of dieldrin (5 μM) and 2,2',4,4',5,5'-HCB (Fig. 4I), as well as DDT (6 μM) and 2,2',4,4',5,5'-HCB (14 μM). Interestingly,
in cells treated with dieldrin, the subcellular localization of gap junction proteins was perinuclear (Fig. 4D).

Using Western blotting, we examined the changes in Cx43 protein levels, as well as the degree of phosphorylation of the Cx43 protein following treatment of the cells with these environmental pollutants. In HBEC, two major bands were detected with a monoclonal antibody specific for Cx43 (Fig. 5B).

Treatment of cell extracts with calf intestinal alkaline phosphatase before protein electrophoresis resulted in the disappearance of the upper band on Western blots (Fig. 5C). This indicated that the upper band was the phosphorylated, and the lower band was the nonphosphorylated species of Cx43.

Cells treated with the vehicle controls (ethanol or DMSO:acetone = 1:1) resulted in a slight reduction of the amount of phosphorylated Cx43 with no apparent loss of GJIC (Fig. 5A) as compared to the untreated controls (Fig. 5B). Cells treated with DDT, dieldrin, toxaphene, and 2,4,5,2',4',5'-HCB at doses which lead to reduced...
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**Figure 5. Western blot analysis of connexin 43 protein in the cell membrane of a normal human breast epithelial cell.** (A) Single treatment: lane 1, vehicle control (ethanol 0.1%); lane 2, vehicle control (dimethylsulfoxide:acetone, 1:1); lane 3, 50 μM DDT; lane 4, 25 μM dieldrin treatment; lane 5, toxaphene (10 μg/ml); lane 6, 56 μM 2,4,5-hexachlorobiphenyl (HCB); lane 7, 70 μM 2,4,5-hexabrominated biphenyl (HBB); (B) Mixture treatment: lane 1, nontreatment control; lane 2, 6 μM DDT; lane 3, 3 μM dieldrin; lane 4, 14 μM 2,4,5-HCB; lane 5, 6 μM DDT + 14 μM 2,4,5-HCB; lane 6, 3 μM dieldrin + 14 μM 2,4,5-HCB; lane 7, 3 μM dieldrin + 8 μM 2,4,5-HBB; lane 8, 8 μM 2,4,5-HBB. (C) Calf intestinal alkaline phosphatase treatment: lane 1, untreated membrane fraction; lane 2, mock treatment without enzyme; lane 3, membrane fraction treated with calf intestinal alkaline phosphatase (10 U/ml); P, phosphorylated band; NP, nonphosphorylated band.

GJIC, display reduced levels of the phosphorylated Cx43 species (Fig. 5A) as compared to vehicle controls. Cells treated with DDT, dieldrin, and 2,4,5,2',4',5'-HBB at doses which do not affect GJIC display levels of phosphorylated Cx43 similar to that of the vehicle controls (Fig. 5B). Cells treated with 2,4,5,2',4',5'-HCB (14 μM) displayed a slight increase in the phosphorylated Cx43 band (Fig. 5B). Treatment of cells with mixtures of DDT (6 μM ) and 2,4,5,2',4',5'-HCB (14 μM), at doses which singly do not affect GJIC, results in further reduction of the phosphorylated Cx43 levels (Fig. 5B) and greatly reduced GJIC. An apparent loss of the phosphorylated Cx43 species was observed in cells treated with a mixture of dieldrin (3 μM) and 2,4,5,2',4',5'-HCB (14 μM; Fig. 5B).

To determine if treatment with these environmental halogenated hydrocarbons altered the level of connexin gene transcripts, the steady-state levels of Cx43 mRNA were analyzed by Northern blotting cells treated with each chemical for 90 min. The data (Fig. 6) showed no significant difference between control cells and these environmental pollutant-treated cells, alone or in mixture.

**Discussion**

It is assumed that inhibition of gap junctional intercellular communication is a mechanistic marker for several toxicological endpoints, such as teratogenesis, tumor promotion, and reproductive and neurological toxicity (20). Therefore, the experiments reported here, using several pollutants known to inhibit GJIC in rodent cells in vitro and to be either teratogens, tumor pro-

mitters, neurotoxicants, or reproductive toxicants in animals (10,17,19), have shown that these chemicals could inhibit GJIC in normal human breast epithelial cells. These studies were prompted by some epidemiological evidence that human exposure to these chemicals via the food chain might be responsible for several human health effects, including breast cancer (21–34).

This study has shown that DDT, dieldrin, toxaphene, and PCB and PBB congeners could inhibit GJIC in normal human breast epithelial cells. Furthermore, these chemicals inhibited GJIC in a reversible manner indicating the effect was not due to a cytotoxic effect and that no-effect or threshold levels existed for each chemical. Interestingly, specific chemicals, which individually did not inhibit GJIC at a given concentration, could, when mixed with another chemical which also did not inhibit GJIC at a certain concentration, interacted to inhibit GJIC.

These studies also demonstrated some differences, as well as some similarities, in the behavior of the normal human breast epithelial cells when compared to rat liver epithelial cells (WB-F344 oval cells). For example, in WB-F344 cells, TPA treatment at a dose level of 10 ng/ml significantly inhibited GJIC (39), but 360 ng/ml TPA had no effect on GJIC in normal human breast epithelial cells (data not shown). On the other hand, DDT, dieldrin, and toxaphene inhibited GJIC in normal human epithelial cells in a manner similar to rat liver WB-F344 cells (39,40). Furthermore, 2,2',4,4',5,5'-HBB and 2,2',4,4',5,5'-HCB inhibited GJIC in normal human breast epithelial cells, whereas coplanar analogs of these chemicals, 3,3',4,4',5,5'-HCB and 3,3',4,4',5,5'-HBB, did not inhibit GJIC at noncytotoxic concentrations. These observations suggest that normal human breast epithelial cells behaved similarly to human teratocarcinoma cells, Chinese hamster V79 cells, and rat liver epithelial cells when tested with these chemicals (41,48–52).

To determine how these chemicals might have inhibited GJIC in these cells, we examined Cx43 protein in cells after treatment with DDT, dieldrin, toxaphene, 2,2',4,4',5,5'-HCB, and 2,2',4,4',5,5'-HBB by immunofluorescent staining. A reduction in the number of gap junction plaques was observed (Fig. 4). Interestingly, in cells treated with dieldrin, the gap junction proteins were observed in the perinuclear area of the cells, suggesting a problem in the trafficking of the gap junction protein. To determine if and how treatment of the normal human breast epithelial cells might affect the phosphorylation status of Cx43, Western blots were performed. The con-
centration of the chemicals that inhibited GJIC indicated alterations in the phosphorylation patterns of these proteins when compared to the control cells (Fig. 6). Several reports have shown that some tumor promoters can induce hyper- or hypophosphorylation of Cx43, which correlated with GJIC (38–40). For example, TPA induced hyper-phosphorylation of Cx43, which altered the subcellular localization of the Cx43 protein under conditions that inhibited GJIC in rat liver WB-F344 and IAR cell lines (38–40).

Immunoblot analyses of Cx43 in normal human breast epithelial cells revealed at least two major protein bands (phosphorylated and nonphosphorylated bands; Fig. 5). The pattern of the different Cx43 bands in normal human breast epithelial cells appears to be different from that seen in normal rat liver WB-F344 cells (unpublished data). DDT, dieldrin, and toxaphene induced hypophosphorylation of Cx43 in normal human breast epithelial cells at concentrations that affected GJIC. There was no change in the steady-state levels of Cx43 mRNA after treatment with these chemicals, alone or in combination (Fig. 6). Comparison of the Cx43 phosphorylation patterns and Cx43 immunostaining in the case of dieldrin mixed with 2,4,5-HCB leads to the hypothesis that 2,4,5-HCB enhances the effects of dieldrin-mediated hypophosphorylation and that 2,4,5-HCB's major effect occurs during plaque internalization. This is proposed because 2,4,5-HCB alone induces little if any Cx43 hypophosphorylation compared to vehicle controls. However, 2,4,5-HCB (GJIC-affecting dose)-treated cells display little Cx43 membrane staining. Dieldrin induces hypophosphorylation and loss of Cx43 membrane plaques (at GJIC-affecting dose). When cells are treated with dieldrin and 2,4,5-HCB mixtures (at GJIC-nonafffecting dose), the phosphorylated species essentially disappears. These results suggest that these environmental halogenated hydrocarbons might alter Cx43 proteins, post-translationally, rather than transcriptionally.

One of the recent concerns of many of the environmental halogenated hydrocarbons is that they might have estrogenic activity, and it is the estrogenlike properties of these chemicals that are responsible for their toxic effects. Soto et al. (22) showed that pesticides such as DDT, dieldrin, and toxaphene are estrogenic in MCF-7 human breast cancer cells. Other groups have suggested a structural relationship to steroid hormones, particularly estrogen and PCB congeners (21). Because humans are exposed to many of these kinds of estrogen-like chemicals (natural and human-made) in their food supply and because several combinations of these chemicals in mixtures can affect GJIC when no single chemical could, the theory that these chemicals might be tumor promoters of human breast cancers could be entertained as possible contributors to the multistage nature of human carcinogenesis.

However, caution must be used in the interpretation of these data. First, human carcinogenesis, like animal carcinogenesis, is a multistep, multimechanism process, involving genotoxic and nongenotoxic mechanisms. The chemicals used in these studies are not genotoxic (40,51) but act via epigenetic mechanisms involving some alterations in signal transduction, such as phosphorylation or dephosphorylation of proteins, ultimately modulating intercellular communication which regulates cell proliferation/differentiation and homeostasis (20). Therefore, these chemicals are probably not tumor initiators but tumor promoters. To be tumor promoters in animals or humans, these chemicals must be present at or above threshold levels to inhibit GJIC and must be acting on preexisting, initiated cells for long periods of time on a regular exposure basis (32).

Recent studies in our laboratory have shown that the normal human breast epithelial cells used (44) in these studies (type II) do not express the estrogen receptor. Therefore, the chemicals used in our studies inhibited GJIC via nonestrogen receptor mechanisms, even though these chemicals are estrogenlike compounds. What is not known is whether an initiated human breast epithelial cell would behave differently from these normal breast epithelial cells when exposed to these chemicals. Future studies will have to be done on normal human breast epithelial cells that express estrogen receptors.

In summary, normal human breast epithelial cells exhibit functional GJIC (44), which can be inhibited by noncytoxic concentrations of DDT, dieldrin, and toxaphene, as well as 2,2,4,4',5,5'-HCB and 2,2',4,4',5,5'-HBB, in a dose-dependent and reversible fashion, with each showing a no-effect or threshold level. Specific mixtures of two of these chemicals showed that, while each alone did not inhibit GJIC, the mixtures did. Therefore, while these chemicals can inhibit GJIC in normal human breast epithelial cells in vitro, it remains to be determined if they can inhibit GJIC in vivo and if these chemicals would affect initiated human breast epithelial cells the same or differently from the way they affect normal cells. Since the inhibition of GJIC has been hypothesized to play a role in the promotion/progression phases of carcinogenesis (35), and since the environmental toxicants used in these studies do inhibit GJIC in animal cells in which they also act as tumor promoters, these studies indicate that these chemicals might exert some human health effects if they meet all the conditions to inhibit GJIC in vivo.

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