Phenolphthalein Exposure Causes Multiple Carcinogenic Effects in Experimental Model Systems

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ABSTRACT

Phenolphthalein (a triphenylmethane derivative) has been commonly used as a laxative for most of the twentieth century, but little is known about its long-term carcinogenic potential in experimental studies. In our studies, phenolphthalein administered continuously in the feed for 2 years to F344 rats at doses of 0, 12,500, 25,000, and 50,000 ppm and to C57BL/6 × CH3 F1 (hereafter called B6C3F1) mice at doses of 0, 3,000, 6,000, and 12,000 ppm caused multiple carcinogenic effects. Treatment-related neoplasms occurred in the kidney and adrenal medulla in male rats, adrenal medulla in female rats, hematopoietic system in male and female mice (histiocytic sarcomas and malignant lymphomas), and ovary of female mice. Phenolphthalein has been shown to have estrogenic and clastogenic properties. Previous studies of other estrogenic chemicals (e.g., zearalenone) in the F344 rat and B6C3F1 mouse have not shown the same spectrum of carcinogenic activity as that found with phenolphthalein, suggesting that phenolphthalein estrogenic activity alone is not responsible for the spectrum of tumors observed. It is more likely that the multiple biological properties of phenolphthalein, including its ability to form free radicals, its clastogenic activity, and its estrogenic activity, contributed to the carcinogenic effects observed. These studies show that phenolphthalein is a multisite/multispecies carcinogen. One of the sites for neoplasms that is of particular concern is the ovary, and epidemiology studies are under way to identify any potential effects of phenolphthalein exposure at this site in humans.

INTRODUCTION

Epidemiological studies have indicated that many human cancers are influenced by environmental factors. Although recent studies have identified genes that are associated with cancer in certain populations, we have not been able to demonstrate that inherited mutations in specific genes are solely responsible for human disease (1). As populations migrate from one area of the world to another, the incidence for a cancer in immigrant populations may approach that of the host country in one or two generations, suggesting that environmental factors may play an important role in the occurrence of some types of cancer (2, 3). Phenolphthalein has been used as an over-the-counter laxative during most of this century (4), and this chemical was studied to determine its potential to cause cancer.

Phenolphthalein has multiple biological properties as demonstrated in various in vitro and in vivo studies. Phenolphthalein was negative for genotoxicity in the Salmonella test both with and without metabolic activation, but was positive in an in vitro chromosomal aberration test conducted with S9 metabolic activation (5). In the B6C3F1 mouse and the Swiss CD-1 mouse, phenolphthalein increases the frequency of micronucleated erythrocytes (6). The lowest dose level at which phenolphthalein causes micronuclei formation is 360 mg/m² body surface area (6), a dose that is approximately two times the recommended dose in humans (185 mg/m² body surface area). Phenolphthalein has been shown to bind competitively to the estrogen receptor in MCF-7 cells (7). Thus, phenolphthalein has the potential to cause adverse biological effects through its clastogenic activity or its estrogenic activity. This article reports the toxic and carcinogenic effects of phenolphthalein in the F344 rat and B6C3F1 mouse after a 2-year exposure period.

MATERIALS AND METHODS

Animals. Male and female F344/N rats and B6C3F1 mice were obtained from Taconic Farms (Germantown, NY) and placed on study at 7–8 weeks of age. Rats were housed by sex, five per cage. Mice were individually housed. Polycarbonate cages (LabProducts, Inc., Rochelle Park, NJ) provided with Sani-chips bedding chips (P. J. Murphy Forest Products, Montville, NJ) were used. Tap water and NIH 07 feed (Zeigler Bros., Gardners, PA) were provided ad libitum. The animals were maintained in a room that was kept at 21–23°C with 10 air changes/h and a 12-h light cycle. All animals were checked daily for clinical signs and moribund animals were necropsied. Animal body weights were taken once a week during the first 13 weeks of study and every 4 weeks thereafter. Feed consumption was recorded weekly.

Chemical. Phenolphthalein (9189; Fig. 1) was supplied by Pharmco Laboratory, Inc. (Tisitville, FL; Fig. 1). The chemical was examined with infrared, UV/visible, and nuclear magnetic resonance spectroscopy and found to be 99% pure by elemental analyses, Karl Fischer water analysis, titration of the functional groups thin-layer chromatography, and high-performance liquid chromatography (National Toxicology Program; Ref. 5). The lot met all USP requirements. Formulated diets were prepared by mixing appropriate amounts of phenolphthalein, a yellow powder, with NIH 07 feed. Periodic analysis of the formulated diets of phenolphthalein were performed, and the diet was found to be within ±10% of the targeted concentrations of 0, 12,000, 25,000, or 50,000 ppm (rats) or 0, 3000, 6000, or 12,000 ppm (mice; Ref. 5).

Two-Year Study Design. Groups of 50 rats and 50 mice of each sex received control or phenolphthalein diets for 104 (males) or 105 weeks (females). A complete gross necropy was performed on all animals from all groups that died or were killed during and at the end of the experiment. All tissues were preserved in 10% neutral buffered formalin. Major organs/tissues trimmed, embedded in paraffin, sectioned, stained with H&E, and examined microscopically included the adrenal gland, bone and marrow, brain, clitoral gland, esophagus, gallbladder, heart, kidney, large intestine (cecum, colon, rectum), liver, lung, mammary gland, mandibular and mesenteric lymph nodes, nose, ovary, pancreas, parathyroid gland, pituitary gland, preputial gland, prostate, salivary gland, skin, small intestine (duodenum, jejunum, ileum), spleen, stomach (fore stomach and glandular), testis with epididymis and seminal vesicle, thymus, thyroid gland, trachea, urinary bladder, uterus, Zymbal’s gland, and all gross lesions.

Statistical Analysis. Differences in survival were analyzed using life table methods (8). For the analysis of tumor incidence data, survival-adjusted procedures were used to assess dose-response trends and to make pairwise comparisons between dose groups and controls (9, 10). Fisher exact tests and Cochran-Armitage trend tests were also utilized to analyze tumor incidence data. Results are considered as significant where the P < 0.05.

RESULTS

Survival, Body Weights, and Clinical Signs. Final survival of exposed rats and mice was similar to the respective controls with the exception of high-dose female mice where survival was less than that of controls. Mean body weights of treated rats were reduced by 5–10% during most of the study, but toward the end of the study the body weight reduction became more pronounced (Table 1).

Clinically, there were no treatment-related signs of toxicity. The
average feed consumption for exposed animals was similar to that of controls. Male rats ate approximately 13—18 g feed/day/animal; female rats ate 9—12 g feed/day/animal; and male and female mice ate 4—5 g feed/day/animal. Based on the feed consumption, dietary levels of 12,000, 25,000, and 50,000 ppm phenolphthalein resulted in average daily doses during the last year of the study of approximately 500, 1000, or 2000 mg phenolphthalein/kg body weight to male rats and 500, 1000, or 2100 mg/kg to female rats. Based on the feed consumption, dietary levels of 3,000, 6,000, and 12,000 ppm phenolphthalein resulted in average daily doses during the last year of the study of approximately 300, 600, or 1,200 mg phenolphthalein/kg body weight to male and female mice (Table 2).

**Treatment-related Lesions: Rats.** There was an increased incidence of proliferative lesions (hyperplasia and pheochromocytomas) in the adrenal medulla of male and female rats (Table 3). Also, the number of males that had pheochromocytomas in both adrenal medullas (bilateral) was increased in the treated groups. Focal hyperplasia was only marginally increased in the treated groups. However, the diagnosis of focal hyperplasia in a gland was only made in the absence of a diagnosis of a neoplasm in that gland. Focal hyperplasia and pheochromocytoma are considered to constitute a morphological continuum in the adrenal medulla.

There was an increased incidence of proliferative lesions of the renal tubule epithelium of all treated groups of male rats. Although the increase was predominantly of hyperplasia and adenoma, carcinoma was slightly increased. Both renal tubular adenoma and carcinoma are relatively uncommon neoplasms in the male F344 rat (i.e., occurring in <1% of untreated control male rats). In the kidney, hyperplasia, adenoma, and carcinoma are thought to represent a continuum in the progression of proliferative lesions of the tubule epithelium.

The incidence of nephropathy of the kidney was increased in all treated female groups, whereas the severity was increased in all treated male groups. Nephropathy is a spontaneously occurring lesion in most aging F344 rats, particularly males, and involves a spectrum of degenerative and associated inflammatory and regenerative changes. Marked tubule dilation with cyst formation and transitional epithelial hyperplasia of the renal pelvis (components of nephropathy) occurred in kidneys from treated males with the most severe nephropathy.

Diffuse hyperplasia of the parathyroid gland, fibrous osteodystrophy of the bone, and mineralization of the glandular stomach (data not shown) were increased in treated groups of male rats. These lesions are commonly observed in rats with severe nephropathy and are associated with a calcium/phosphorous imbalance created by compromised functional capacity of the kidney.

**Treatment-related Lesions: Mice.** An increased incidence of histiocytic sarcoma was observed in male and female mice (Table 4). Spontaneous histiocytic sarcoma occurred in 0.5—1.0% of the untreated B6C3F1 mice. In this study, histiocytic sarcoma was consistently observed in the liver with several other sites (e.g., spleen, lung, bone marrow, and various lymph nodes) less frequently involved.

Exposure to phenolphthalein was associated with an increased incidence of malignant lymphoma (all sites) and lymphoma of thymic origin in all exposed groups of female mice. The incidences of lymphoma of thymic origin were increased in exposed groups of male mice, but were significantly increased only in the 6000 ppm group. Atypical hyperplasia of the thymus was also observed in treated males and females. Lymphomas were considered of thymic origin if (a) they were observed only in the thymus and other observed proliferative lymphocytic lesions were limited to the thoracic cavity or (b) the thymus clearly contained the largest proliferative lymphocytic lesion when systemic lymphocytic lesions occurred.

In the mouse, the thymus is a bilobed organ, with each lobe having a distinct outer cortex composed of small hyperchromatic lymphocytes and an inner medulla composed of larger lymphocytes and fewer epithelial cells. In some treated mice, one or both of the lobes lacked the normal corticomedullary arrangement. When these abnormal lobes were less than the size of a normal thymic lobe and were comprised of sheets of large lymphocytes admixed with variable numbers of smaller more normal-appearing lymphocytes, the change was diagnosed as atypical hyperplasia. The incidence of atypical hyperplasia of the thymus was also increased in treated males and females. In the earliest lesions diagnosed as malignant lymphoma, a fairly homogeneous population of lymphocytes extended beyond the confines of normal thymic tissue and mitoses were common. Larger thymic lymphomas occupied a large portion of the chest cavity.

There was an increased incidence of benign sex-cord stromal ovarian tumors and associated stromal cell hyperplasias in all treated female groups. The neoplasms were discrete masses which varied from occupying most of the ovary to markedly expanding it (up to eight times) with compression of surrounding parenchyma. Neoplasms and hyperplasias were usually composed of sheets of round to oval stromal cells with abundant cytoplasm, which varied from finely granular and eosinophilic to vesiculated (leuteinized). In most neoplasms the cells were rather homogeneous with a low mitotic index; however, in some of the larger ones there was a modest degree of cellular pleomorphism with a higher mitotic rate.

Proliferative lesions (neoplasms and foci) of the liver were decreased in treated male and female mice. Furthermore, many of the hepatocellular adenomas in the control groups were multiple, but not in treated groups. Hepatocellular neoplasms occur with a high and variable rate in the B6C3F1 mouse [with a range of 10—68% in untreated male mice (mean 41%) and a range of 6—42% in untreated female mice (mean 21%)]. Although there is a known association of the incidence of this neoplasm with body weight in the B6C3F1,

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**Table 1  Survival and body weight**

<table>
<thead>
<tr>
<th>Dose (ppm)</th>
<th>0</th>
<th>12,000</th>
<th>25,000</th>
<th>50,000</th>
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</thead>
<tbody>
<tr>
<td>Male rats</td>
<td>Survival</td>
<td>21/50</td>
<td>15/50</td>
<td>25/50</td>
</tr>
<tr>
<td></td>
<td>Final mean body weight (%)</td>
<td>84</td>
<td>79</td>
<td>79</td>
</tr>
<tr>
<td>Female rats</td>
<td>Survival</td>
<td>34/50</td>
<td>41/50</td>
<td>37/50</td>
</tr>
<tr>
<td></td>
<td>Final mean body weight (%)</td>
<td>86</td>
<td>86</td>
<td>85</td>
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</table>

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<table>
<thead>
<tr>
<th>Dose (ppm)</th>
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<th>300</th>
<th>6,000</th>
<th>12,000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male mice</td>
<td>Survival</td>
<td>40/50</td>
<td>33/50</td>
<td>36/50</td>
</tr>
<tr>
<td></td>
<td>Final mean body weight (%)</td>
<td>97</td>
<td>99</td>
<td>93</td>
</tr>
<tr>
<td>Female mice</td>
<td>Survival</td>
<td>42/50</td>
<td>33/50</td>
<td>34/50</td>
</tr>
<tr>
<td></td>
<td>Final mean body weight (%)</td>
<td>92</td>
<td>95</td>
<td>94</td>
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* Percentage relative to controls.
mouse, the slight decreases in body weights of treated groups could not account for the decreased incidences observed in this study.

In addition to the neoplastic effects, there were a number of nonneoplastic effects considered related to administration of phenolphthalein in mice. These included mild to moderate testicular degeneration in males which involved germinal epithelium, increased incidence of myelofibrosis of the bone marrow of males, and an increased incidence of degeneration of the teeth in treated males, and an increased incidence of spleen hematopoietic cell proliferation in males.

**DISCUSSION**

At the end of the 2-year study period, phenolphthalein caused neoplasia in the ovary of female mice, the hematopoietic system of male and female mice, the adrenal of male and female rats, and the kidney of male rats.

The phenolphthalein-induced ovarian neoplasms were considered to be of sex cord/stromal origin rather than epithelial or germ cell. Like many cancers, ovarian cancers develop from nonneoplastic effects considered related to administration of phenolphthalein for 2 years. In almost all instances, these have been mixed tumors of sex cord/stromal origin rather than epithelial or germ cell. The adrenal cortex of male and female rats, and the bone marrow of males, and an increased incidence of degeneration of the teeth in treated males, and an increased incidence of spleen hematopoietic cell proliferation in males.

**Table 2 Summary of feed and compound consumption by rats and mice in the 2-year phenolphthalein study**

<table>
<thead>
<tr>
<th>Dose (ppm)</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>12,000</td>
<td>25,000</td>
</tr>
</tbody>
</table>

**Table 3 Treatment-related lesions in rats exposed to phenolphthalein for 2 years**

<table>
<thead>
<tr>
<th>Tumor site</th>
<th>Dose (ppm)</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>12,000</td>
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</table>

**Male rat**

<table>
<thead>
<tr>
<th>Tumor site</th>
<th>Dose (ppm)</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>12,000</td>
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**Female rat**

<table>
<thead>
<tr>
<th>Tumor site</th>
<th>Dose (ppm)</th>
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</thead>
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<tr>
<td>0</td>
<td>12,000</td>
</tr>
</tbody>
</table>

**Cancer of the ovary is the fourth most common cancer in American women. There was an estimated 26,600 new cases diagnosed in 1995 and 14,500 deaths resulted from this disease** (13). In humans, approximately 80% of the ovarian tumors are of epithelial origin; other ovarian tumors may be of sex cord/stromal origin. Like many cancers, ovarian cancer is a disease of aging, with almost one half of new cases occurring in women age 65 years or older.

Environmental factors that might contribute to ovarian cancer in humans have not been identified. It is known that the rate for ovarian cancer in American women is two to three times higher than that for Orientals (2). It is not known whether this difference in the ovarian cancer rate is due to environmental or genetic factors. BRCA1 and BRCA2 mutations have been associated with familial cancer but only 5–10% of ovarian carcinomas are thought to result from hereditary predispositions (14), and for the large majority of human ovarian cancer cases examined, mutations in the BRCA1 or BRCA2 locations have not been observed (14–16).

Like phenolphthalein, which has been shown to bind competitively to the estrogen receptor in MCF-7 cells (7), other chemicals tested in the bioassay have also been shown to interfere with estrogen binding at the estrogen receptor and to have mitogenic effects on breast cancer cell lines (17). These include zearalenone, 2,4-dichlorophenol, and butyl benzyl phthalate (11, 17). However, of these chemicals, only phenolphthalein was shown to cause ovarian neoplasms in mice. Thus, demonstration of a chemical-induced estrogenic response does not necessarily correlate with ovarian or mammary gland neoplasia in rodents. Furthermore, these chemicals with estrogenic activity show different neoplasm patterns in other tissues. This suggests that phenolphthalein study(11). Thus, in our model systems, the mouse is more sensitive to a chemically induced ovarian tumor response than the rat.
nolphthalein-induced neoplasms may be due to multiple factors, which might include specific metabolism and distribution of the chemical as well as estrogenic or genotoxic properties of the chemical.

The hematopoietic system of the mouse was affected by phenolphthalein exposure. There was a clear increase in the incidence of histiocytic sarcomas and malignant lymphomas in all groups of male and female treated mice. Chemical-associated increases in histiocytic sarcoma are not commonly observed in the bioassay in mice. The incidence of spontaneous histiocytic sarcoma occurs two to three times more frequently in female than in male B6C3F1 mice; however, in this phenolphthalein study, incidences were greater in the treated male groups. Histiocytic sarcomas were observed most often in the liver and occasionally at other sites. Histiocytic sarcoma are generally considered to arise from a macrophage/histiocyte and possibly from the specialized Kupffer cell of the liver, but definitive data relative to the site of origin are lacking.

Many of the phenolphthalein-induced malignant lymphomas were thought to originate in the thymus. Spontaneously occurring lymphomas in mice in the NTP studies usually originate in the spleen or lymph nodes rather than in the thymus. With phenolphthalein, 1,3-butadiene, and DDC (18), the lymphomas originated primarily in the thymus.

Phenolphthalein, 1,3-butadiene, and DDC have in common positive estrogenic activity of phenolphthalein, including the testicular degeneration and a decrease in tooth dysplasia in treated male mice. Estrogen has been shown to inhibit liver tumor promotion in mice (25), and, in NTP studies, spontaneous liver tumors occur in males at twice the rate of females. In this study, the rates for liver tumors were markedly decreased in both sexes of mice.

The incidence of pheochromocytoma of the adrenal medulla was significantly increased in all groups of treated male rats and in low- and mid-dose female rats. The mechanism for the formation of this tumor is not known and has been previously observed with both genotoxic and nongenotoxic chemicals (11). Of approximately 250 NTP studies in which chemicals were administered in the feed to rats, phenolphthalein is the only chemical that caused adrenal tumors in both male and female rats. The carcinogenic effect at the same site in both sexes reinforces the treatment-related carcinogenic response at this site.

The kidney tumors observed in phenolphthalein-treated male rats may have been related to toxic effects of the chemical at this site. There was an increase in the severity of nephropathy in all treated rat groups, and the severity was greater in males. There was an increase in the renal tubule proliferative lesions, including hyperplasia, adenoma, and carcinoma in treated male rat groups.

The findings in the male rat kidney are similar to those observed with other cyclic lactone chemicals, including quercetin, coumarin, and dihydrocoumarin (26). With these chemicals, there was no morphological evidence of toxicity to the kidney early in the study, but toward the end of the 2-year study the severity of nephropathy was increased in male rats (and to a lesser extent in female rats) and a few kidney tumors occurred in the male rat. The greater sensitivity of the male rat to this kidney toxicity is apparently due to a greater susceptibility of male rats to spontaneous nephropathy during aging and the exacerbation of this disease by chemical administration.

There is no readily apparent common mechanism by which these renal proliferative lesions developed. One possible mode of action is consistent with the theory of increased cell replication providing a fertile ground for increased mutation rates and neoplasm development. Renal cellular damage is thought to increase the amount of renal tubule epithelial regeneration via cell replication. In one study, [1H]thymidine labeling demonstrated increased levels of DNA synthesis to be directly proportional to the increased severity of nephropathy in aging female F344/N rats (27).

Phenolphthalein is absorbed in the small bowel and is conjugated in the liver to form phenolphthalein glucuronide, which is eliminated in the bile; as it passes through the small intestine it is partially deconjugated and reabsorbed (28, 29). The major metabolite of phenolphthalein is the glucuronide. The plasma level of free phenolphthalein...
PHENOLPHTHALEIN EXPOSURE IN EXPERIMENTAL MODELS

is approximately 0.1–0.2 μg/ml plasma over a dose range of 500–2000 mg/kg/day. The findings that the plasma level of free phenolphthalein were approximately the same for the three dose levels used in the 2-year study may explain in part why the carcinogenic response was not proportional to dose in the kidney of male rats, ovary of female mice, and hematopoietic system of mice. The low dose used in the 2-year mouse study is within 10 times the human dose level when compared on a mg/m² body surface area basis (Table 2).

Other structurally related di- or triphenylmethylene chemicals (e.g., gentian violet) form free radicals (30, 31) and cause cancer in mice [hematological cancers and ovarian toxicity (32)]. Recent studies have also confirmed that phenolphthalein forms free radicals, suggesting that phenolphthalein may be a significant source of oxidative stress in physiological systems. It is possible that one mechanism for the cancer seen in the phenolphthalein-treated animals is through the formation of oxygen radicals and subsequent cellular damage (33). Phenolphthalein is capable of being converted to a quinoid structure. Quinoids are highly reactive chemicals capable of reacting with sulphydryl groups and amino groups and forming free oxygen radicals (34).

Epidemiology studies are now underway to identify any potential ovarian effects that phenolphthalein exposure may have on women in the United States.

ACKNOWLEDGMENTS

We thank Dr. Joel Mahler and Dr. Thomas Burk, National Institute of Environmental Health Sciences and Dr. Michael Elwell, Experimental Pathology Laboratories for their helpful comments and review of the manuscript.

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