A Rat Mammary Tumor Model Induced by the Organophosphorous Pesticides Parathion and Malathion, Possibly through Acetylcholinesterase Inhibition

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Environmental chemicals may be involved in the etiology of breast cancers. Many studies have addressed the association between cancer in humans and agricultural pesticide exposure. Organophosphorous pesticides have been used extensively to control mosquito plagues. Parathion and malathion are organophosphorous pesticides extensively used to control a wide range of sucking and chewing pests of field crops, fruits, and vegetables. They have many structural similarities with naturally occurring compounds, and their primary target of action in insects is the nervous system; they inhibit the release of the enzyme acetylcholinesterase at the synaptic junction. Eserine, parathion, and malathion are cholinesterase inhibitors responsible for the hydrolysis of body choline esters, including acetylcholine at cholinergic synapses. Atropine, a parasympatholytic alkaloid, is used as an antidote to acetylcholinesterase inhibitors. The aim of this study was to examine whether pesticides were able to induce malignant transformation of the rat mammary gland and to determine whether alterations induced by these substances increase the cholinergic activation influencing such transformation. These results showed that eserine, parathion, and malathion increased cell proliferation of terminal end buds of the 44-day-old mammary gland of rats, followed by formation of 8.6, 14.3, and 24.3% of mammary carcinomas, respectively, after about 28 months. At the same time, acetylcholinesterase activity decreased in the serum of these animals from 9.78 ± 0.78 U/mL in the control animals to 3.05 ± 0.06 U/mL; 2.57 ± 0.15 U/mL; and 3.88 ± 0.44 U/mL in the eserine-, parathion-, and malathion-treated groups, respectively. However, atropine alone induced a significant ($p < 0.05$) decrease in the acetylcholinesterase activity from the control value of 9.78 ± 0.78 to 4.38 ± 0.10 for atropine alone, to 1.32 ± 0.06 for atropine in combination with eserine, and 2.39 ± 0.29 for atropine with malathion, and there was no mammary tumor formation. These results indicate that organophosphorous pesticides induce changes in the epithelium of mammary gland influencing the process of carcinogenesis, and such alterations occur at the level of nervous system by increasing the cholinergic stimulation. *Key words*: acetylcholinesterase, atropine, malathion, parathion, rat mammary cancer. *Environ Health Perspect* 109:471–479 (2001). [Online 3 May 2001]
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Environmental chemicals may be involved in the etiology of breast cancers. Many human tumors have been causally attributed to exposure to environmental carcinogens, pollutants, pesticides, drugs, ultraviolet light, radiation, and tobacco (*1*). The incidence of breast tumors in women is increasing, and environmental chemicals have been partially implicated in this increase (*2*). *In vivo* and *in vitro* data have shown that environmental substances (e.g., DDT, polychlorinated biphenyls, 4-nonylphenol, 4-octylphenol) can promote mammary cancer (*3,4*).

The toxic effect of organophosphorous insecticides, which represent a major class of agricultural chemicals, is to conjugate with the natural complement of cholinesterase enzymes in the body, thereby inactivating them. Parathion [0,0-diethyl 0-(4-nitrophenyl)-phosphorothyoate] and malathion [0,0-dimethyl *S*-(1,2-dicarbethoxy-ethyl)-phosphorodithioate] are organophosphorous pesticides that are extensively used to control a wide range of sucking and chewing pests of field crops, fruits, and vegetables. They have structural similarities with naturally occurring compounds, and their primary target of action in insects is the nervous system. Malathion is also present in lotions and shampoos marketed for the treatment of head lice and mites in humans. Exposure of the skin to these two pesticides has been shown to result in a small amount of systemic absorption (*5,6*).

Eserine [(3α,*S*-*cis*)-1,2,3,3α,8,8α-Hexahydro-1,3α,8-trimethylpyrrolo (2,3-*b*) indol-5-ol methylcarbamate] is an ester obtained from calabar beans, the seeds of the vine *Physostigma venenosum*. It is used as a miotic drug and for atony of gastrointestinal tract, but it is very toxic if inhaled or swallowed (*5*). Eserine, as well as parathion and malathion, is incorporated through the epithelium of the skin, mouth, and respiratory tract (*5,6*). In the liver, parathion and malathion are activated by enzymatic processes producing paraoxon and malaoxon, respectively (*5,6*). Eserine, parathion, and malathion are acetylcholinesterase (AchE) inhibitors and are responsible for the hydrolysis of body choline esters, including acetylcholine (Ach) at cholinergic synapses (*5–7*). The inhibition of these enzymes increases the availability of Ach, which in turn can stimulate cholinergic receptors producing both nicotinic and muscarinic effects in the organism such as muscle contractions and secretions in many glands (*5*). Atropine is a parasympatholytic alkaloid used as an antidote to AchE inhibitors (*5,6*); it acts through competitive occupation of nicotinic and muscarinic cholinergic receptors (*8*).

The high level of cell proliferation and differentiation that occurs during mammary gland development makes this organ an attractive experimental animal model for examining its susceptibility to different carcinogenic actions. Cell proliferation in the mammary gland is not a random event, but is intimately related to both topography of the mammary parenchyma and specific stages of the gland development that are modulated by age, hormonal variations, and parity history. Different compartments, such as ducts, ductules, and intralobular terminal ducts, have been observed in rats. The intralobular terminal duct is equivalent to the terminal ductal lobular unit in the human breast, considered the site of origin of human breast carcinomas (*9–14*).

The mammary gland is a complex organ that undergoes continuous changes under the influence of body growth as well as cyclic hormonal stimulation from birth to senescence. Under these influences, the

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histology of the gland becomes extremely heterogeneous. The rat mammary glands are distributed in pairs along the milk line, with one pair located in the cervical, two pairs in the thoracic, one in the abdominal, and two in the inguinal region. The basic development of all the glands follows a uniform pattern, and previous studies (*9*) have established criteria for evaluating mammary growth, development and differentiation. With such criteria, the mammary parenchyma is divided into thirds along the longitudinal axis. The third closest to the nipple, called zone A, is composed of the main lactiferous ducts. The intermediate area, called zone B, contains more abundant lateral buds, and it is in this area where most of the secondary ducts arise. The distal third of the gland, or zone C, contains the terminal structures ending in bulbous clubs or terminal end buds (TEBs) (*9*). The mammary gland is composed of a single primary or main lactiferous duct that branches into three to five secondary ducts at birth and during the first week of postnatal life. The ducts are narrow and straight and end in small, club-shaped terminals, the TEBs. During the second week, further sprouting of ducts occurs up to the sixth cell generation. This sprouting of ducts causes a marked increase in the density of TEBs, which reaches its maximum magnitude when the animal is 21 days old (*9*). Histologically, TEBs are composed of three to six layers of medium-sized epithelial cells. After the rat reaches 21 days of age, further sprouting of lateral buds occurs, and numerous TEBs begin to cleave into three to five smaller buds, the alveolar buds (ABs). The differentiation of the mammary gland induces a progressive decrease in the number of TEBs and a concomitant increase in the number of ABs (*9–13*).

The crucial role that topography plays in susceptibility of the breast to carcinogenesis in young nulliparous rats involves the presence of TEBs. The TEBs are the most highly proliferating structures in the breast and therefore are the most susceptible to neoplastic transformation (*9–13*). The study of the pathogenesis of mammary cancer in experimental models has shown that the susceptibility of the mammary gland to chemical carcinogenesis is directly related to the rate of cell proliferation of the glandular epithelium at the time of the exposure to the carcinogen (*11*).

The most widely used experimental systems for the study of mammary tumorigenesis are the models in which tumors are induced in the Sprague-Dawley rat by dimethylbenz[α]anthracene (DMBA) (*15–17*) or in the Sprague-Dawley or Fischer 344 rat by *N*-nitrosomethylurea (NMU) (*18*). The administration of DMBA to virgin rats of different ages induced tumors with an incidence that was directly proportional to the density of highly proliferative TEBs (*12*). Tumor incidence of 94–100% was obtained when DMBA was administered to rats 30–55 days of age; but the highest number of tumors per animal was observed when the carcinogen was given to animals when they were 40–46 days of age—a period when TEBs were most actively differentiating into ABs (*9–13*).

Many studies have addressed the association between cancer in humans and agricultural pesticide exposure, both occupational (e.g., farmers, pesticides mixers, and appliers) and nonoccupational. Nonoccupational exposures occur in farmers exposed to pesticides stored in homes, contaminated clothing, household dust containing pesticides, contaminated ground and surface water, contaminated soil, and drift from aerial

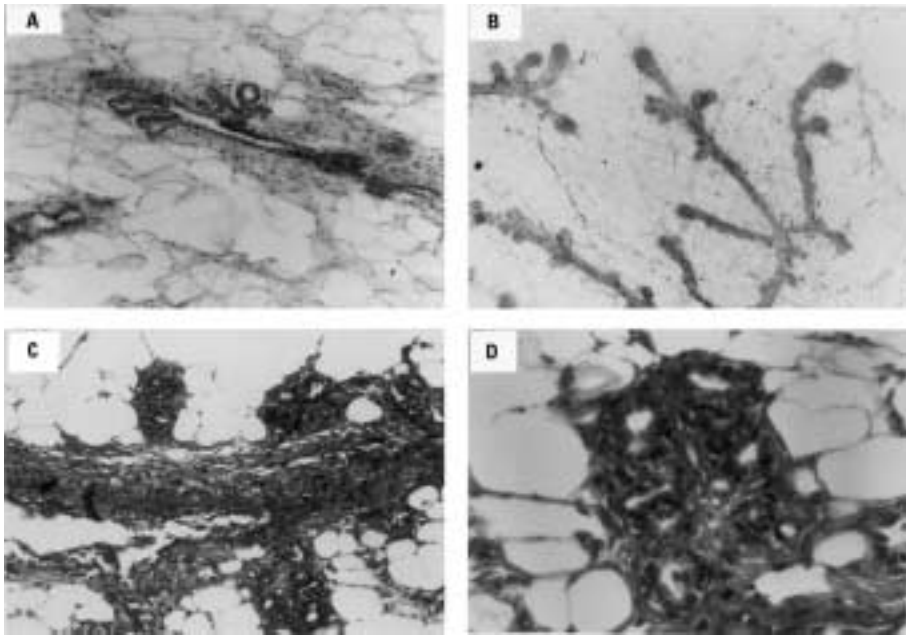

Figure 1. Structures of a normal rat mammary gland composed of a main lactiferous duct (*A*; 350×), which begin to cleave into smaller TEBs and ABs forming lobules (*C*). (*B*) Whole-mount preparation of representative TEBs. (*D*) Higher magnification of a lobule formed by ABs found in cross-section of (*C*). Hematoxylin-eosin, 400×.

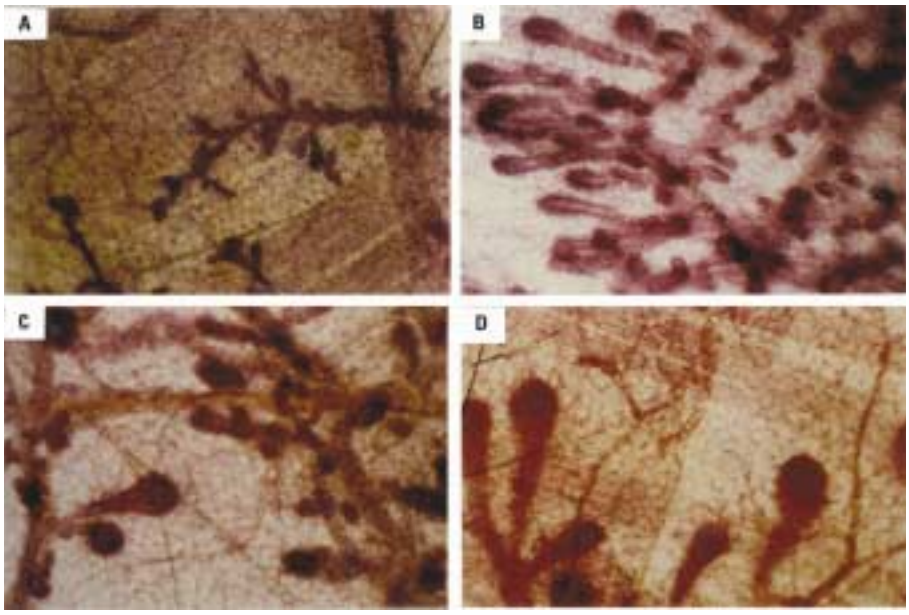

Figure 2. Whole-mount preparation of the abdominal (fourth pair) 21-day-old female rat mammary gland. Presence of TEBs in (*A*) control, (*B*) eserine-, (*C*) parathion-, and (*D*) malathion-treated animals. Hematoxylin-eosin, 400×.

spraying of pesticides. Mortality and incidence studies have reported slightly increased rates for the following cancers: breast, non-Hodgkin lymphoma, Hodgkin disease, multiple myeloma, leukemia, prostate, and ovary (*19–21*).

Our purpose was to test the susceptibility of the mammary gland to organophosphorous pesticides. Therefore, we analyzed whether eserine, parathion, and malathion can induce mammary tumors in rats, whether the alterations induced by these substances alter the cholinergic activation influencing malignant transformation of the mammary gland, and whether such action can be inhibited by a parasympatholytic alkaloid, such as atropine.

Materials and Methods

Female Sprague-Dawley rats were obtained from the Catholic University of Chile (Santiago, Chile). These animals were housed and bred in a barrier animal facility operated in accordance with the standards outlined in *Guide for the Care and Use of Laboratory Animals* (*22*). All animals were allowed continuous access to a standard laboratory chow diet (Champion, Santiago, Chile). The first experiment was performed with 35 virgin female Sprague-Dawley rats 16 days old. Seven groups of five animals received injections twice a day for 5 days subcutaneously (sc) or intraperitoneally (ip) in the inguinal region of the body. Animals were injected with one of the following subtances: saline solution (sc); eserine (sc; Sigma, St. Louis, MO, USA), 33 µg/100 g body weight (bw); parathion (sc; Bayer, Santiago, Chile), 250 µg/100 g bw; malathion (sc; Fyfanon TM, Cheminova, Denmark), 17 mg/100 g bw; atropine (ip; Sigma), 250 µg/100 g bw; combination of eserine and atropine; and combination of malathion and atropine at the dosages previously indicated. The LD_{50} values of the substances for eserine, parathion, and malathion were 0.64, 3.6, and 1,000 mg/kg, respectively. However, the doses used in these experiments were one-half the LD_{50} for eserine and parathion and one-sixth the LD_{50} for malathion, which allowed a 100% survival of animals after 5-day treatment.

The second experiment was performed under identical conditions but the experiment started when the animals were 39 days old. Sixteen hours after the last injections, the animals from these two experimental designs were anesthetized by ip injections of sodium pentobarbital (8 mg/100 g bw) and opened by a midline incision from the pubis to the submaxillary area to remove the mammary glands. The skin was dissected to expose the six pairs of mammary glands, the thoracic, abdominal, and inguinal mammary glands. The left side of control and treated rats was prepared for whole-mount preparation and the right side for histologic studies.

Mammary glands for the whole-mount studies were fixed in alcohol, formaldehyde, and acetic acid (1:1:1) for 24 hr at room temperature, stained with hematoxylin-eosin, and mounted between two slides to visualize under a Zeiss Video Plan (Chicago, IL, USA) (*10*). The mammary gland of rats is sufficiently flat to allow the determination of the area and the quantification of structures in areas of 1 mm^2. We counted 50 areas located mainly distal to the nipple in each gland, and we counted the density of TEBs and ABs in these whole-mount preparations. The density of these structures was expressed in number of structures per square millimeter. To determine the histology, we fixed the glands in Bouin's fixative and embedded them in

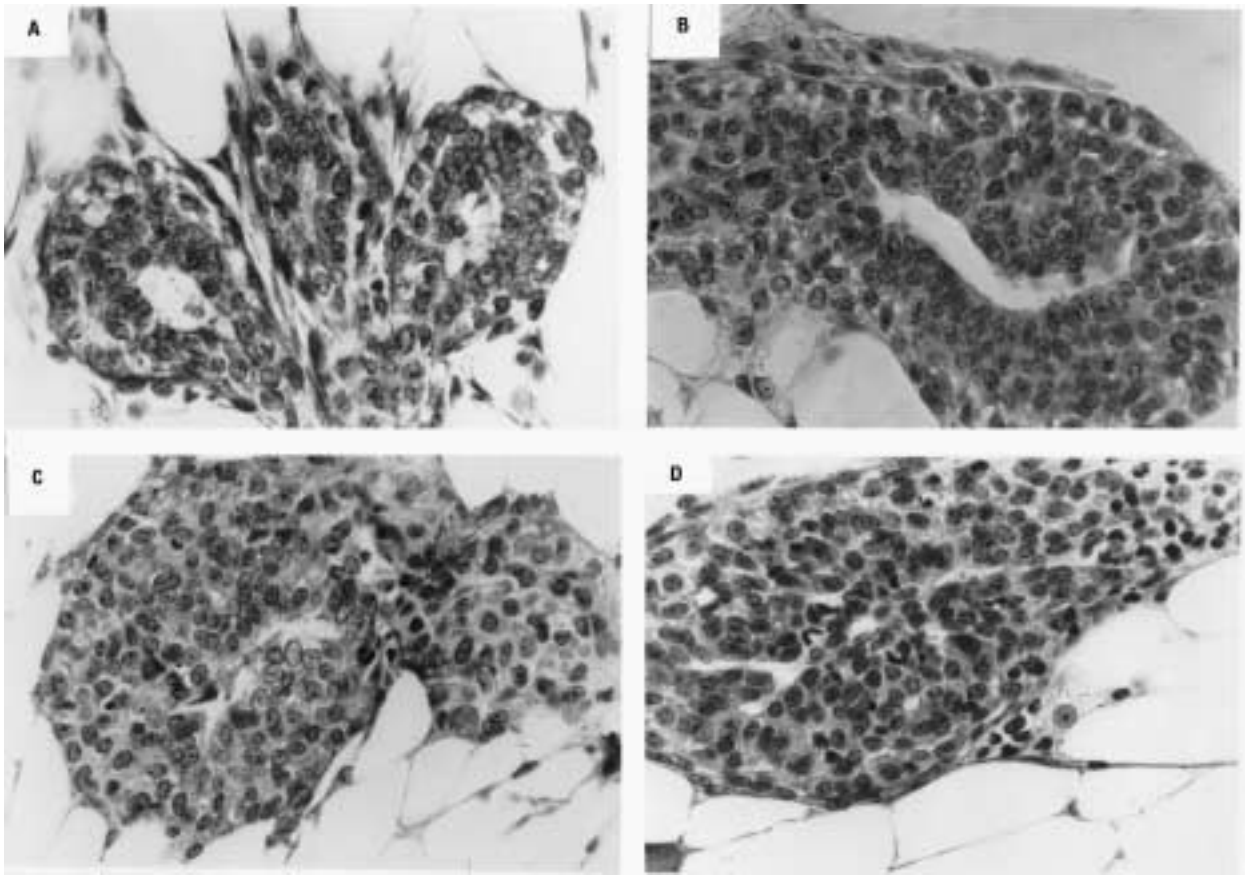

Figure 3. Histologic section of TEBs of the mammary gland from the control and 21-day-old treated female rats. Presence of TEBs in (*A*) control, (*B*) eserine-, (*C*) parathion-, and (*D*) malathion-treated animals. Hematoxylin-eosin, 400×.

paraffin. The glands were oriented flat so that the parallel sections corresponded with the whole-mount preparation. Then the glands were serially sectioned at a thickness of 5 µm and stained with hematoxylin-eosin. Histomorphometry was performed using a Bausch & Lomb (Rochester, NY, USA) binocular microscope, in which a 1-mm^2 grid was installed in one of the oculars. The measurements were done with 10× ocular. We counted five slides per animal to determine the number of TEBs and ABs in the mammary gland from control and treated animals. A total of 20 areas of 1 mm^2 were counted in each gland. The data are expressed as average ± standard error (SE) of the mean.

We used blood extracted directly from the heart of the animals to determine the AchE activity in the serum. Whole blood was centrifuged at 2,500 cycles per min; serum was analyzed with the Caraway spectrophotometric method in the presence of 0.1 mM ethopropazine (Sigma), a substance that inhibits butyrylcholinesterase activity (*23*). One unit of activity is equal to 1 µmol of substrate breakdown at 25°C. The results are expressed as average ± standard error of the mean. Statistical analysis was performed with the *F*-test and all pairwise multiple comparison procedures (Tukey *t*-test) between groups with significance at the $p < 0.05$ level.

The third experiment was performed with 560 virgin female rats at 39 days of age. Eight groups of 70 animals each received sc injections (except for atropine, which was given ip twice a day for 5 days) in the inguinal region of one of the following substances: saline solution; eserine; parathion; malathion; atropine; combination of eserine and atropine; and combination of malathion and atropine at dosages previously indicated. All the animals were housed three per cage for 28 months and were palpated weekly to detect formation of tumors. After a tumor was detected, the data were recorded and tumors were allowed to develop for 1 month. After that time the animals were sacrificed. Palpable tumors were removed from the animal, fixed with Bouin's fluid, and embedded in paraffin. The blocks were sectioned at a thickness of 5 µm. Deparaffinized sections were stained with hematoxylin-eosin, and the tumors were analyzed under the microscope as previously described (*9*,*14*).

Results

The structure of a normal rat mammary gland is composed of a single primary or main lactiferous duct that branches into secondary ducts from which TEBs and the alveolar buds (ABs) are formed. The ducts are narrow and straight and end in small, club-shaped terminal structures, the TEBs (Figure 1A,B), composed of three to six layers of medium-sized epithelial cells from which numerous TEBs begin to cleave into smaller buds, the ABs from which lobules are formed (Figure 1C,D). Analysis of normal rat mammary glands indicates that density of TEBs (number of TEBs per square millimeter) increased steadily after birth and reached its maximum value when animals were 21 days of age. A 21-day-old rat mammary gland is made up mainly of TEBs. Figure 2 shows the presence of TEBs and absence of

Table 1. Effect of eserine, parathion, malathion, and atropine on density of TEBs and ABs of 21-day-old female Sprague-Dawley rat mammary glands (mean ± SE, $n = 5$)

Treatment	TEBs/mm^2	AB/mm^2
Control	9.06 ± 0.97	0.06 ± 0.05
Eserine	7.49 ± 0.93	0.00 ± 0.00
Parathion	7.53 ± 0.30	0.00 ± 0.00
Malathion	7.08 ± 0.37	0.00 ± 0.00
Atropine	9.44 ± 0.90	0.00 ± 0.00
Atropine + eserine	9.80 ± 1.90	0.00 ± 0.00
Atropine + malathion	10.14 ± 1.80	0.00 ± 0.00

Density of TEBs and ABs of mammary gland of whole-mount preparations of animals that received twice a day for 5 days injections of saline (control); 33 µg of eserine; 250 µg parathion; 17 mg malathion; 250 µg atropine; or combinations of atropine + eserine or atropine + malathion per 100 g body weight. There was no significant difference in TEBs or ABs between control animals and those treated with eserine, parathion, malathion, atropine, atropine + eserine, or atropine + malathion.

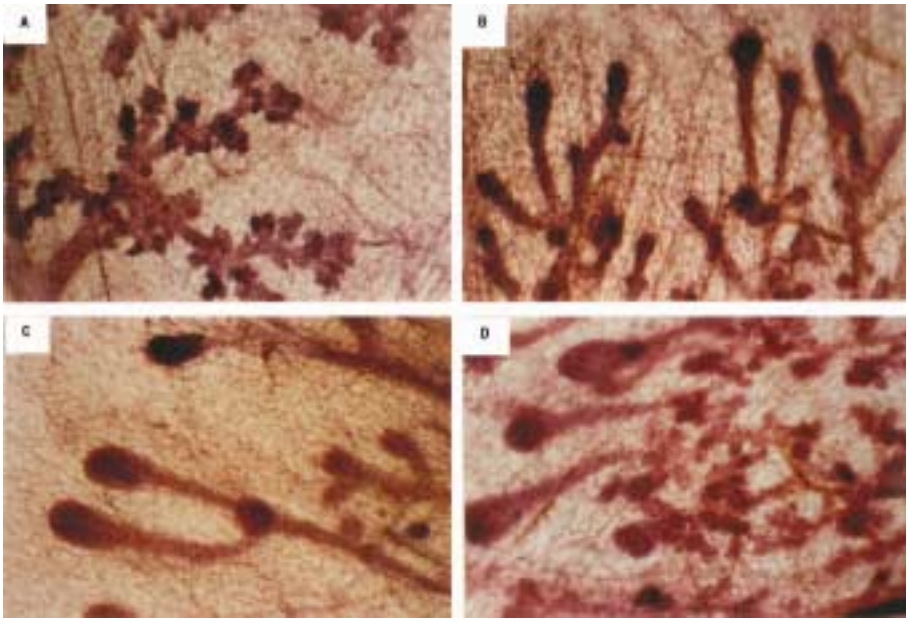

Figure 4. Whole-mount preparation of the mammary gland of 44-day-old animals. It shows the presence of ABs in (*A*) control, and the presence of TEBs in the (*B*) eserine-, (*C*) parathion-, and (*D*) malathion-treated animals. Hematoxylin-eosin, 400×.

Table 2. Effect of eserine, parathion, malathion, atropine, and combination of either atropine + eserine or atropine + malathion on density of TEBs and ABs of 44-day-old female Sprague-Dawley rat mammary glands (means ± SE, $n = 5$)

Treatment	TEBs/mm^2	AB/mm^2
Control (saline)	3.30 ± 0.27	20.80 ± 1.68
Eserine (33 µg/100 g bw)	11.13 ± 1.67	0.75 ± 0.44
Parathion (250 µg/100 g bw)	12.04 ± 1.77	1.28 ± 0.52
Malathion (17 mg/100 g bw)	11.26 ± 0.48	2.50 ± 0.56
Atropine (250 µg/100 g bw)	3.48 ± 0.42	5.60 ± 0.57
Atropine + eserine	4.50 ± 0.75[a]	16.00 ± 0.80[b]
Atropine + malathion	1.82 ± 0.43[a]	14.96 ± 1.35[b]

Density of TEBs and ABs in whole-mount preparations of animals that received injections twice a day for 5 days. TEBs: $p < 0.05$: control versus eserine, parathion, and malathion. TEBs: $p < 0.05$: Eserine versus atropine + eserine and malathion versus atropine + malathion.
[a]TEBs: nonsignificant: atropine versus atropine + eserine and versus atropine + malathion. ABs: $p < 0.05$: control versus eserine, parathion, malathion, and atropine. [b]ABs: nonsignificant: control versus atropine + eserine or atropine + malathion.

ABs in the control (Figure 2A), eserine- (Figure 2B), parathion- (Figure 2C), and malathion- (Figure 2D) treated 21-day-old animals. Figure 3 shows a cross-section of mammary glands of the same animals, which corroborated the presence of TEBs in the control (Figure 3A), eserine- (Figure 3B), parathion- (Figure 3C), and malathion- (Figure 3D) treated animals. These results indicated that there was no significant difference in the density of TEBs per square millimeter of mammary gland between either the control or the eserine-, parathion-, and malathion-treated 21-day-old rats, and that ABs were absent in the control as well as in the treated animals (Table 1).

Whole-mount preparations in 44-day-old rats showed a significant ($p < 0.05$) difference in the density of TEBs and ABs between control (Figure 4A) and treated animals (Figure 4B–D). Table 2 shows that treatment with eserine and the pesticides parathion and malathion induced a significant increase ($p < 0.05$) in the density of TEBs in the 44-day-old animals. The TEB density increased from 3.30 ± 0.27 TEBs/mm^2 to a maximum of 12.04 ± 1.77 TEBs/mm^2 in parathion-treated animals (Table 2). The AB density decreased in treated animals from 20.80 ± 1.68 AB/mm^2 in controls to a minimum of 0.75 ± 0.44 AB/mm^2 in eserine-treated animals (Table 2). Parathion- and malathion-treated animals also showed a significant ($p < 0.05$) decrease in the density of ABs. A representative whole-mount preparation of a 44-day-old rat mammary gland of control animals and a schematic representation of ABs are presented in Figure 5A and B, respectively. Representative whole-mount preparation from a pesticide-treated rat mammary gland and a schematic representation of such structures, which gives an overview of TEBs, are shown in Figures 5C and D, respectively.

Histologic examination of mammary tissue of 44-day-old control and treated rats supported the results seen with the whole-mount preparation. The control rats had normal lobule formation and presence of ABs, whereas no ABs were observed in any group of treated animals (Figure 6A–D). The eserine-, parathion-, and malathion-treated animals showed a significant ($p < 0.05$) increase in the size of TEBs of the mammary gland, as well as in the number of the epithelial layers (Figure 6B–D). Furthermore, such structures increased in size until tumors started to appear. However, after 840 days the control animals did not develop any kind of tumors. After pesticide injections, treated animals developed tumors at a rate of 8.6–24.3%. Table 3 shows the number, size, and latency of these tumors after 28 months of treatment. Among the 560 animals used in this experiment, 6 out of 70 eserine-treated animals, 10 out of 70 parathion-treated animals, and 17 out of 70 malathion-treated animals developed mammary tumors. Tumor size ranged from 9.26 ± 3.43 to 22.1 ± 7.2 cm^3. The malathion-treated group had smaller tumors, but they appeared earlier than with the other treatments. The tumor latency range fluctuated between 54 and 840 days. Treated animals exhibited only mammary tumors that appeared exclusively in the mammary fat pad of the axillary and abdominal-inguinal regions of the animal (Figure 7A,B). Histologic diagnosis of the mammary tumors revealed that they were adenocarcinomas, grossly nodular and encapsulated, with areas of cribiform or papillary patterns (Figure 7C, D). Most of the tumors developed in the abdominal region, with only one tumor in the cervical region. Analysis of lungs, heart, intestinal tract, ovaries, and uterus did not show any tumors. Neurologic symptoms that might have suggested tumors of the nervous system were not observed.

Tables 1 and 2 also show the effect of atropine either alone or in combination with eserine or malathion on the density of structures present in the rat mammary glands. Neither atropine nor the combination of atropine with eserine or malathion induced changes in the density of TEBs per square millimeter in the mammary gland of 21-day-old animals in comparison to control animals (Table 1). There was no significant difference among these groups in comparison to control animals. Table 2 shows the density of TEBs and ABs in the mammary gland of 44-day-old animals treated with atropine alone or in combination with the eserine or malathion. Treatment of animals at this age with atropine alone or in conjunction with eserine or malathion did not induce any change in the density of TEBs in comparison to the control animals (Figure 8A). However, atropine caused a significant ($p < 0.05$) inhibition of formation of ABs in these animals compared to controls. Atropine treatment in combination with either eserine or malathion caused a significant ($p < 0.05$)

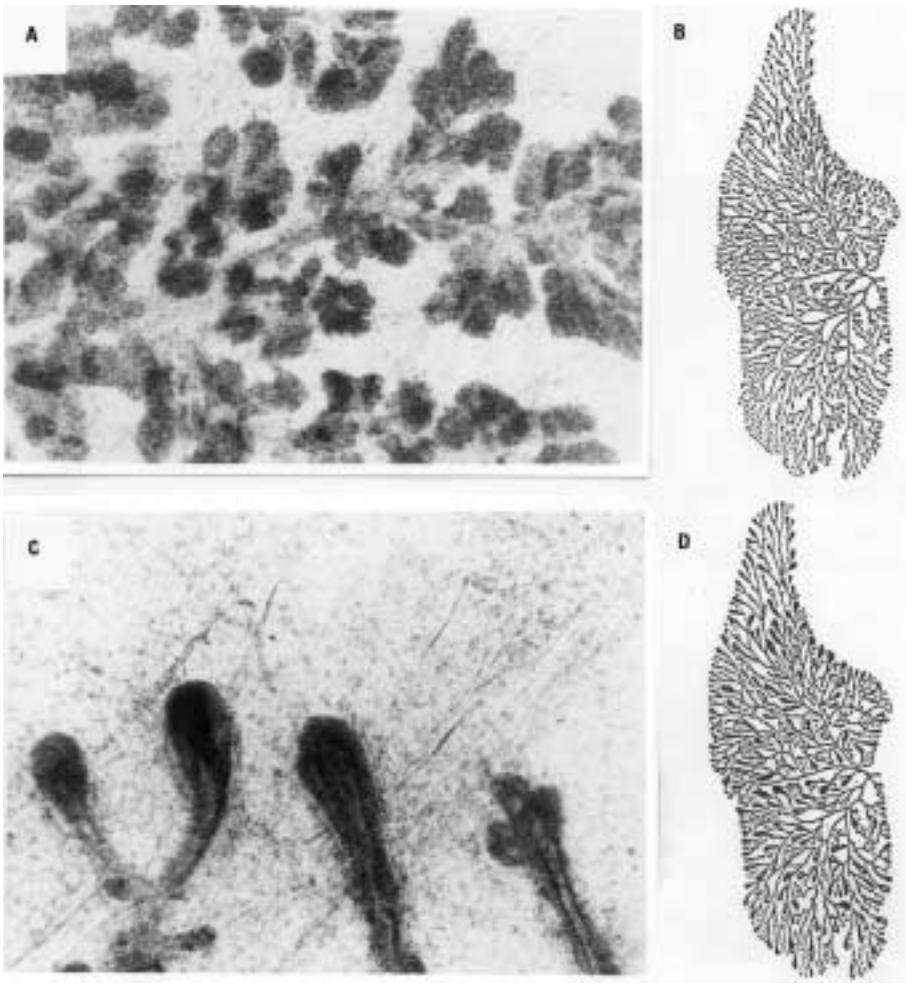

Figure 5. Whole-mount preparation of the mammary gland. (*A*) ABs; (*B*) a schematic representation of these structures; (*C*) TEBS in pesticide-treated animals; and (*D*) an overview of (*C*). 400×.

inhibition of density of TEBs in comparison with the eserine, parathion, and malathion alone. However, there was an increase in the density of ABs similar to that of controls (Figure 8B) and a significant ($p < 0.05$) increase in the density of ABs in comparison to animals treated with eserine, parathion, and malathion. In addition, there was no rat mammary tumor formation after the injection of atropine alone or with the combination of atropine and eserine or malathion. Figure 9 shows the correlation between the density of TEBs and AchE activity in the serum of the 44-day-old control and treated animals. These results showed a significant ($p < 0.05$) decrease in the AchE activity in serum of eserine, parathion, malathion, atropine, and combination of atropine either with eserine- or malathion-treated animals in comparison to controls. The atropine induced an even greater decrease ($p < 0.05$) in such activity from 4.38 ± 0.2 U/mL in the atropine-treated group to 1.32 ± 0.02 and 2.39 ± 0.2 when combined with the eserine and malathion, respectively.

Discussion

Our study demonstrates that parathion and malathion induced tumor formation in a specific target organ, the mammary gland. This is the first report that organophosphorous pesticides induce changes in mammary gland associated with carcinogenesis. Our results showed that initiation occurs primarily in the epithelium of TEBs while they are developing into ABs because these structures were affected by pesticides. This is an important finding because such structures are considered equivalent to the terminal ductal lobular unit described in the human breast (*9–13*). Treatment with these substances induced significant changes at a cellular level. Forty-four-day-old animals showed a significant increase in the density of TEBs, inhibiting the normal process of differentiation from TEBs to ABs that was seen in the control animals. These results indicated that the proliferative changes observed in the TEBs may have induced the formation of mammary adenocarcinomas.

Breast tissue seems to be very sensitive to ionizing radiation when exposure occurs between the ages of 15 and 19 years (*9*), which suggests that events that occur during the early years of a woman's life have a significant effect on lifetime risk of breast cancer. An understanding of the mechanisms that make the mammary glands of young women more susceptible to carcinogenesis can be achieved by using an adequate animal model system. Previously, researchers (*10,15*) have demonstrated that mammary carcinoma formation in rats can be induced by a chemical carcinogen, mainly DMBA, which seems to provide such a model. Mammary tumors thus induced were adenocarcinomas arising from TEBs of incompletely differentiated glands (*10,12*). The administration of DMBA to virgin rats of different ages induced tumors with an incidence that was directly proportionally to the density of highly proliferating TEBs (*10*). A tumor incidence of 94–100% was obtained when DMBA was administered to rats 30–55 days of age, but the highest number of tumors per animal was observed when the carcinogen was given to animals when they were 40–46 days of age, a period when TEBs were most actively differentiating into ABs. The high susceptibility of the TEBs to neoplastic transformation is attributed to the cell kinetic properties of its lining epithelium, whose rate of cell proliferation is shown by DNA synthesis. The TEBs were also characterized by having a high growth fraction, proliferative fraction of the cell cycle, which diminished in the more differentiated ABs and lobules (*9–13*).

To determine whether cell kinetic parameters varied among the different compartments of the breast in the human female and whether they were affected by age and gland topography (*24*), samples of normal human breast tissue were studied in organ culture systems. The results indicated differences among the mammary glands of young and old women. The correlation of different parameters related to cell cycle determination indicated that values for growth fraction and DNA synthesis or DNA-labeling index were higher in the intralobular terminal ducts and lower in alveoli and ducts. Studies done on the influence of age and gland topography on cell kinetics of normal human breast indicated that both young and

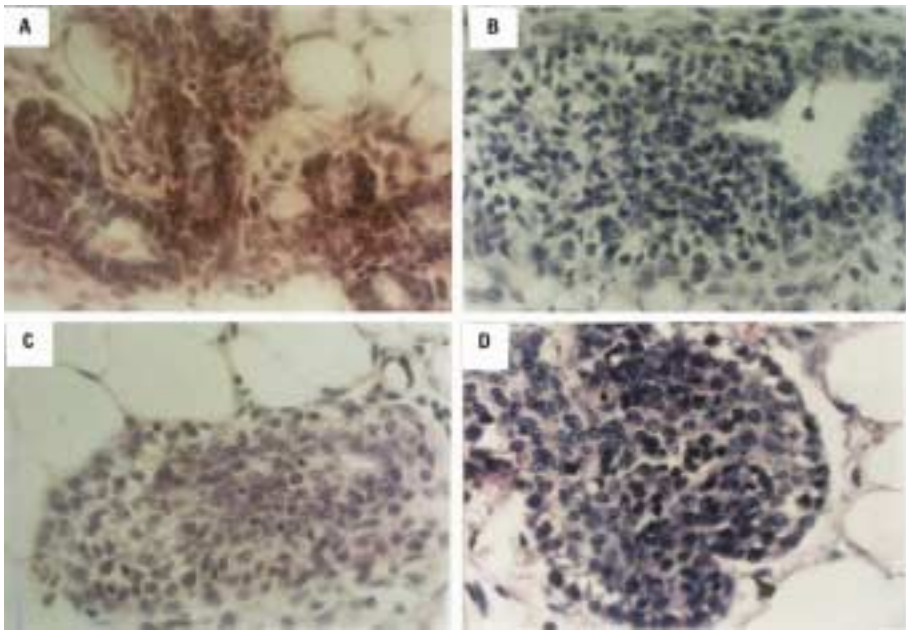

Figure 6. Histologic section of 44-day-old female rat mammary gland. (*A*) Histologic section of ABs of the mammary gland from the control animal. The proliferative characteristics of TEBs in (*B*) eserine-, (*C*) parathion-, and (*D*) malathion-treated animals are appreciated here. Hematoxylin-eosin, 400×.

Table 3. Analysis of mammary gland tumor formation by the effect of eserine, parathion, malathion, atropine, and combinations of eserine + atropine, parathion + atropine, and malathion + atropine in female Sprague-Dawley rats treated from 39 to 44 days of age.

Treatment	No. animals with tumors/total animals	Mean tumor size ± SE (cm^3)[a]	Mean latency ± SE (days)[b]
Control (saline)	0/70	—	—
Eserine (33 µg/100 g bw)	6/70	22.10 ± 7.20 (5.4–49.56)	488 ± 60 (402–840)
Parathion (250 µg/100 g bw)	10/70	16.50 ± 5.60 (2.5–45.00)	569 ± 16 (490–619)
Malathion (17 mg/100 g bw)	17/70	9.26 ± 3.43 (0.08–42.30)	(509 ± 45) (54–653)
Atropine (250 µg/100 g bw)	0/70	—	—
Atropine + eserine	0/70	—	—
Atropine + parathion	0/70	—	—
Atropine + malathion	0/70	—	—

[a]Range in tumor size. [b]Range of number of days before tumors appeared.

older women had the highest growth fraction and DNA-LI in the intralobular terminal ducts. The length of the cell cycle in these structures increased from 200.3 to 847.0 hr in the older women (*24*). The high rate of cell proliferation was associated with the shortened length of the cell cycle. Other reports (*9–13*) have shown that DNA-LI among young virgin rats was much higher than among old virgin rats. The DNA-LI studies in young women paralleled those results. The TEBs of young virgin rats had a cell cycle of an average length of 11 hr, lengthening to 21 and 28 hr in the terminal ducts and ABs, respectively.

According to previous observations, the susceptibility of the mammary gland to neoplastic transformation is related to its degree of development and proliferative activity (*25*). *In vitro* oncogenesis models of different types of cells have yielded important knowledge about the pathogenesis of neoplasia. Attempts to obtain transformation of human breast epithelial cells in culture have been also successful (*25–27*). Miyamoto et al. (*27*) observed neoplastic transformation of mouse mammary epithelial cells by *in vitro* exposure to NMU. McCormick et al. (*28*) reported neoplastic transformation of epithelial cells by the effect of NMU in mammary gland *in vivo*. The formation of tumors observed in eserine-, parathion-, and malathion-treated animals correlates with the greater density of TEBs in the mammary gland present in the 44-day-old treated animals. The concomitant increase in the area of ABs in the control animals and lack of tumors in control animals allows corroboration of our results with what other groups have found (*12*).

Models for studying mammary carcinogenesis have been developed in Sprague-Dawley rats, with the chemical carcinogen DMBA (*15–17*), which induced mammary carcinomas in 100% of the animals with a latency period of 86 days (*15*). Another widely used experimental system to study mammary tumorigenesis is the model in which tumors are induced in the Fischer 344 rats by a single dose of NMU; such tumors had latency similar to that observed by the group mentioned above (*18*). In contrast to these potent carcinogens, which induce mammary carcinomas in 100% of intact females (*14*), organophosphorous pesticides seem to have a slow and less infiltrating and potent effect. We observed that parathion and malathion induced 14.3% and 24.3% of mammary carcinomas, respectively. The type of tumors observed had papillary adenomatous patterns and ductal carcinomas with cribiform pattern.

Mammary tumors formed in various strains of rats induced by chemical carcinogens or radiation have been classified by several authors (*9,14,29*). There is agreement that tumors appearing histologically malignant in the rat have features in common with most frequently observed tumors in humans. Intraductal and infiltrating ductal carcinomas in the rat, unlike in the human, constitute a minority of the tumors developed under the commonly used regimens for tumor induction. Most tumors induced in young virgin rats can be classified within three major groups: intraductal

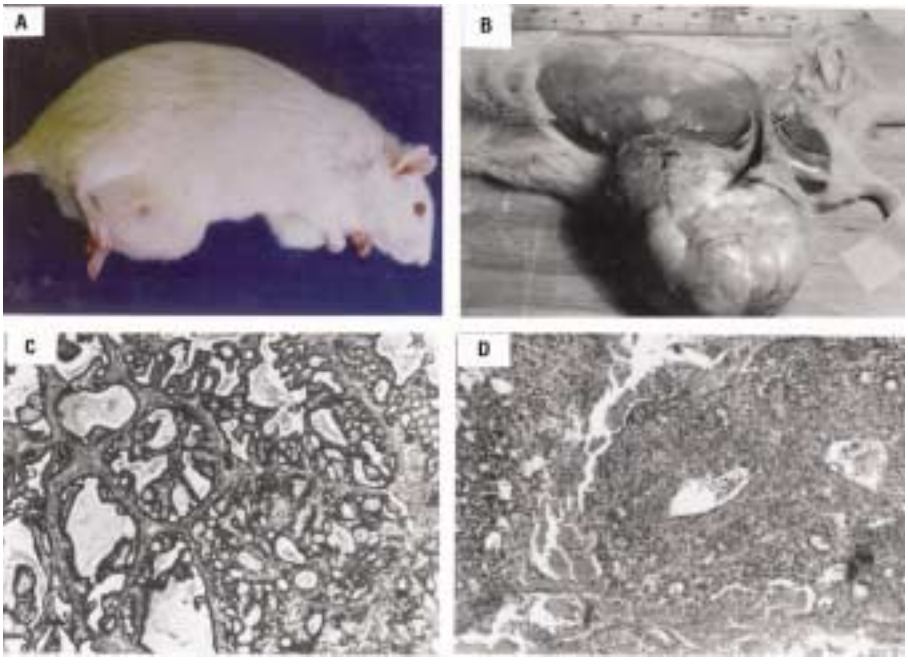

Figure 7. Mammary gland tumors developed in the mammary gland region by the effect of (*A*) parathion and (*B*) malathion in the female Sprague-Dawley rats. (*C*) Cross-sections of a papillary adenocarcinoma, grossly nodular, encapsulated, with areas of cribiform or papillary patterns. It was removed after 84 days of 5-day malathion treatment. (*D*) The histologic sections of another mammary tumor removed after 96 days of 5-day malathion treatment that revealed a ductal carcinoma. Hematoxylin-eosin, 400×.

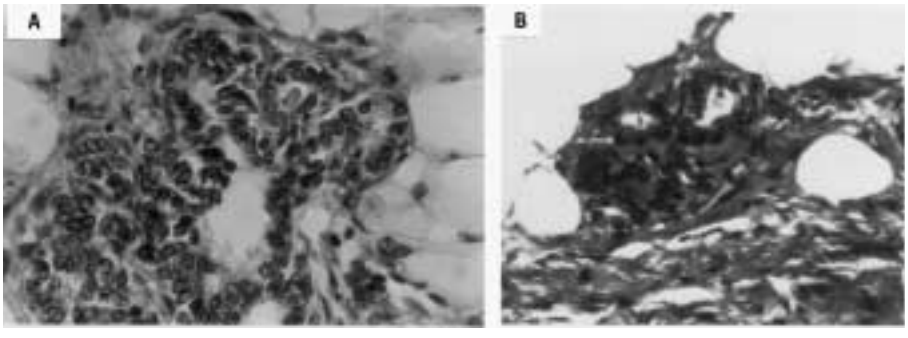

Figure 8. (*A*) Cross-section of structures present in the mammary gland of a rat injected with atropine (400×). (*B*) Cross-section of ABs of a rat treated with the combination of atropine and pesticide. Hematoxylin-eosin, 400×.

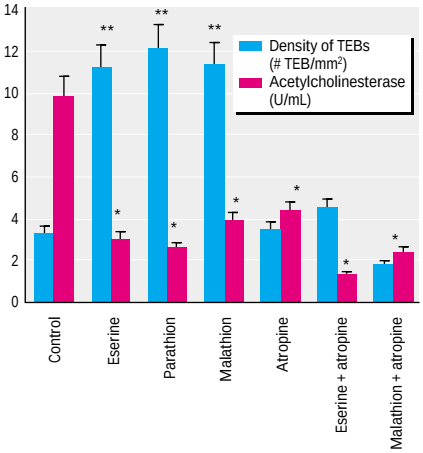

Figure 9. Correlation between the density of TEBs of the mammary gland and the AchE activity (U/mL) present in the serum of 44-day-old control and treated animals. The bars represent the mean ± SE of five animals per group.
*$p < 0.05$ versus control; **$p < 0.05$ versus control.

papillomas, composed of a hyperplastic epithelium; papillary carcinomas, which may be composed of epithelial cells with varying degrees of cytologic atypia, growing in solid, papillary adenomatous patterns; and ductal carcinomas with cribiform pattern. All three kinds of tumors were observed in the eserine-and pesticide-treated animals.

The density of TEBs was almost 4 times greater, and the density of ABs was 10 times lower in the pesticide-treated 44-day-old animals than in the control ones. Even though the pesticides seem to act on undifferentiated structures (i.e., TEBs), they also seem to have a different mechanism of action, probably through the inhibition of AchE that increases Ach availability. For that reason, the incidence of such tumors seems to be slower through the inhibition of AchE than with the direct-acting carcinogen DMBA. Our results confirmed this interaction because 8.6% of the animals treated with eserine also had mammary gland tumors.

Atropine alone did not induce changes in the cell proliferation of mammary gland of 44-day-old rats compared to the control, whereas atropine induced inhibition in formation of ABs compared to the controls. However, the atropine treatment in conjunction with either eserine or malathion produced a decrease in the density of TEBs and an increase in the formation of ABs similar to control animals. On the other hand, we also observed that atropine treatment either alone or in combination with eserine or malathion decreased three to five times the AchE activity in the serum of animals, compared to controls. The ABs reached the normal development of the mammary gland similar to the control animals, and there was no tumor formation. The interesting finding of these studies was that mammary tumors were found only in the eserine-, parathion-, and malathion-treated animals. Our experiments indicated that the pesticides induced AchE inhibition which remained for at least 3 months, suggesting that there was constant cholinergic stimulation. It has been previously reported that animals treated with AchE inhibitors presented a higher tolerance to cholinergic agonists (*5,6*). Under normal physiologic conditions, the cholinergic receptors are usually operative through Ach action, and atropine is known to occupy the cholinergic receptors and interfere with the action of Ach (*5,6*). Therefore, atropine may inhibit the stimulatory effect of pesticides on the Ach receptor.

Previous work (*30*) has shown that a single injection of isoproterenol increases incorporation of thymidine into DNA of salivary gland of rats and mice. This substance is a β-catecholamine adrenergic agonist that is believed to induce chemical changes in the cell membrane implicated in the control of cell proliferation. Hypertrophy and hyperplasia of parotid salivary gland has been indicated by DNA synthesis. Other reports (*31*) have indicated that reserpine, a drug that alters the autonomic nervous system by depleting the noradrenergic endings, also induces remarkable changes in the salivary gland. We have previously shown that eserine, which acts on the autonomic nervous system, can induce changes in DNA synthesis of rat submandibular gland after a 5-day *in vivo* treatment (*32*), which confirmed the proliferative effect of this substance.

Parathion is highly toxic to mammals, and the LD_{50} values in rats have been reported to be 3.6 mg/kg, and the acute oral LD_{50} values were 13 mg/kg (*33,34*). The dosage of parathion in these studies was low (250 µg/100 g bw); therefore, prolonged exposure to low doses of pesticides may eventually cause serious health effects in the population. Among other factors that may be involved with pesticide-induced mammary carcinogenesis is the metabolism of the organophosphorous insecticides, because they can undergo a variety of metabolic transformations *in vivo* and *in vitro* (*35*). Thus, although the original insecticides are poor inhibitors of AchE, the oxygen analogs are several orders of magnitude more potent in their inhibition of the target enzyme, AchE. Benke and Murphy (*36*) have reported pathways of metabolism that may contribute to age differences in toxicity of pesticides. A gradual decrease in the susceptibility to toxicity by parathion with increasing age was observed in rats. Because of changes in the rates of reactions in the detoxification pathways for methyl paraoxon and paraoxon rather than in the metabolism of the original insecticides, the animals became less sensitive to the acute effects of these two substances with increasing age. Brodeur and Dubois (*37*) found that weaning rats 23 days old were more susceptible than adult animals to organophosphorous pesticides, implying that age is another factor in pesticide exposure.

We can conclude that the mechanism of action of parathion and malathion occurs probably through the inhibition of AchE, the enzyme responsible for the hydrolysis of Ach at cholinergic synapses (*5,6*) as it occurs with eserine treatment. Thus, alterations at the nervous system level seem to increase the cholinergic stimulation and probably alter the molecular pathways that initiate the proliferation leading to mammary carcinogenesis. Because parathion and malathion have been used extensively in Latin American countries as well as in many other countries, these studies are relevant to understanding the possible effects of these agents.

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