ABSTRACT

Trifluralin, a widely used herbicide, added to the diet before the p.o. administration of benzo(a)pyrene (BP) and fed continuously, significantly inhibited the induction of lung and forestomach tumors in female A/J mice. Dietary intake of trifluralin before the administration of BP resulted in a significant increase in glutathione in lung and forestomach but not in liver and glandular stomach. Trifluralin treatment also inhibited the binding of [3H]BP to liver and lung DNA, as well as to protein in the liver. Under these conditions, the protection against BP-induced lung tumors and perhaps forestomach tumors may be due to an elevation of tissue glutathione, resulting in a decreased binding of reactive metabolites of BP to macromolecules at these sites. The results indicate that trifluralin has a "blocking" effect in its inhibition of BP-induced tumors.

Our studies show that trifluralin also inhibits chemical carcinogenesis in lung and forestomach when started in the diet 1 day after the administration of BP and fed continuously thereafter. In the case of lung, although maximum inhibition of tumors occurred when trifluralin was started 1 day after BP, there was significant protection at all time intervals (0 to 7 days) against lung tumors. The finding that trifluralin protects against BP tumorigenesis when started in the diet after the administration of the carcinogen clearly demonstrates that trifluralin also has a "suppressive" effect against BP-induced tumors.

INTRODUCTION

The human population is chronically exposed to low levels of herbicides and carcinogenic hydrocarbons, such as BP. The dinitroaniline herbicides are an important group of compounds of which the use is expanding. The most widely used member of this group is trifluralin (27, 32). Numerous studies have established that BP is metabolized by the tissue microsomal monooxygenase enzyme system (cytochrome P-450 or P-448 enzymes) to become reactive electrophilic epoxides that bind covalently with proteins and intracellular nucleic acids in target organs for the formation of tumors (18, 33). Cytochrome P-450-mediated enzymes (15), microsomal enzymes such as epoxide hydrase (15, 23), UDP glucuronosyltransferases (2), and soluble enzymes such as sulfotransferases (2, 8) and GSH transferases (11), can be responsible for detoxification of BP. In lung, the alveolar type II cell from which BP tumors develop has been reported to contain the enzyme BP hydroxylase, also called a cytochrome P-450 mixed-function oxidase enzyme (13). The cytochrome P-450 enzyme system which metabolizes BP also metabolizes trifluralin (7, 20). Emmerson and Anderson (7) have identified 10 different metabolites of trifluralin in rats.

The induction of activating and detoxifying enzymes by environmental chemicals, such as trifluralin, can alter the host response to other foreign compounds, including carcinogens. We have reported previously that trifluralin protected against BP-induced pulmonary and forestomach tumors in female A/J mice (34). An important mechanism of the protection against chemical carcinogenesis may involve conjugation of reactive electrophilic metabolites of carcinogens with GSH. Tissue GSH is critically involved in the interaction of reactive BP electrophiles with nuclear DNA (5, 6). The antioxidant butylated hydroxyanisole has been reported to elevate tissue GSH and GSH transferase activity in target organs where protection was observed against BP-induced tumors (31, 37, 39).

The interaction of trifluralin on BP carcinogenesis has been studied using a lung and forestomach tumor model described by Wattenberg (36). In the present study, the effects of trifluralin on BP hydroxylase activity, GSH levels, and binding of BP to macromolecules (DNA, RNA, and protein) were also determined.

These biochemical studies were conducted in target organs for tumor development and nontarget organs to help elucidate possible mechanisms involved in the observed protection by trifluralin against BP-induced tumors.

MATERIALS AND METHODS

Chemicals. Practical-grade BP (minimum purity, 95%) was obtained from Sigma Chemical Co., St. Louis, MO. Other chemicals received from Sigma included GSH, o-phenthaldehyde, sodium-p-aminosalicylate, RNase (bovine pancreas type IA), and RNA (calf liver type IV). Trifluralin, Lot 326EF8, was donated by the Elanco Products Division of Lilly Research Laboratory, Greenfield, IN. [G-3H]BP (specific activity, 37 Ci/mmol or 146 mCi/mg) was obtained from Amersham, Arlington Heights, IL. Radioactive purity determined by thin-layer chromatography on silica gel in n-hexane was estimated to be greater than 98%. The radioactive concentration was 5 mCi/ml toluene. Radioactive and nonradioactive BP in corn oil were mixed to give the specific activity desired. Sodium trispropylarylphthalate sulfonate, orcinol, phenol, m-cresol, 8-hydroxyquinoline, m-phosphoric acid, and N-ethylmaleimide were received from Fisher Scientific Co., King of Prussia, PA. Diaminobenzolic acid dihydrochloride (99%) was obtained from Aldrich Chemical Co., Milwaukee, WI. Bovine serum albumin was obtained from Miles Laboratory, Inc., Elkhart, IN; Soluene 350 and Dimilume 30 were from Packard Instruments, Downers Grove, IL; and DNA (calf thymus) and DNase (Code DPF) were from Worthington Biochemical Corp., Freehold, NJ. Corn oil, Mazola brand, was bought in a local grocery store.

Animals. All animals used were female A/J mice purchased from the Jackson Laboratory, Bar Harbor, ME, and allowed to acclimate for 1 week.

Diets. Control and experimental diets containing trifluralin were pre-
TRIFLURALIN INHIBITION OF BENZO(A)PYRENE TUMORIGENESIS

pared by Dyets, Inc., Bethlehem, PA.

Treatment with Trifluralin before BP. At 9 weeks of age, these mice were weighed and placed in 3 groups, one control and 2 experimental receiving trifluralin. Each animal was administered 3 mg of BP in 0.25 ml of corn oil (p.o.) on the seventh day (10 weeks of age) and 21st day (12 weeks of age) following the start of trifluralin feeding.

Treatment with Trifluralin after BP. Animals were administered 3 mg of BP in 0.25 ml of corn oil (p.o.) at 6 and 8 weeks of age. Control and trifluralin diets were started at varying time intervals (0, 1, 4, and 7 days) after the last dose of BP. Mice from each of the paired-control and trifluralin-fed groups were sacrificed for tumor analysis after 12 weeks on the trifluralin diet.

Tumor Determination. Animals were sacrificed by cervical dislocation. Lungs were removed and placed in Tellyesniczky's acetic biochromatic solution for 2 days and examined grossly. Lung tumors appeared as pearly white nodules, while normal tissue was stained darker. The presence of stomach tumors was determined as described by Wattenberg (36). Tumors measuring 1.0 mm or larger were counted and assessed microscopically for tumor type. The relative susceptibility to BP-induced tumors is expressed by the "tumorigenic index" as proposed by Shimkin (30).

Binding of p.o. Administered [3H]BP to Macromolecules. At 9 weeks of age, mice were fed a control diet and 2 levels of trifluralin. On the seventh day, the mice (10 weeks of age) were administered their first p.o. dose of BP in 0.25 ml of corn oil. On the 21st day, at 12 weeks of age, they were given 150 mg of BP with 15 ml of [3H]BP per kg p.o. in corn oil at 1% of body weight. Mice were sacrificed 24 hr after the administration of [3H]BP. Liver and lung were removed, rinsed in cold 1.15% KCl, and frozen in liquid nitrogen. Organs were wrapped in aluminum foil and stored at -70° until processed for the determination of DNA and RNA by modification of the method of Lijinsky and Ross (16).

Organs were weighed in the frozen state, and a 10% homogenate was made in the prescribed homogenizing solution. This homogenate was tested to determine [3H]BP binding to protein by the method of Tuné et al. (35) as follows: 0.5 ml of the homogenate was treated with 3 ml of cold 10% trichloroacetic acid to precipitate proteins. Proteins were pelleted by spinning at 1000 x g for 10 min. The pellet was washed successively as follows: 3 washes with alcohol:ether (3:1); one wash with acetone:hexane (2:5:1); and 5 washes with methanol. The pellet was then dissolved in 1.0 ml of 1.0 M NaOH by heating to 90° for 10 min. The assay for protein was done by the method of Lowry et al. (17).

For determination of DNA and RNA, a 5.0-ml aliquot of homogenate was treated with an equal volume of Kirby phenol mixture.

The RNA precipitate obtained by centrifugation was washed twice with 2% sodium acetate in 75% ethanol, once with 95% ethanol, once with absolute ethanol, once with ethanol:ether (1:1), and once with ether. The precipitate was then dissolved in 1.0 ml of 0.05 M magnesium sulfate in 0.1% sodium perchlorate. Incubation with 0.1 ml of DNase (25 µg/ml) at 37° for 30 min was followed by precipitation of RNA with 2 volumes of 95% ethanol. The precipitate was then dissolved in 1.0 ml of 1.0 M sodium perchlorate, extracted with chloroform:isoamyl alcohol (24:1), and reprecipitated with 2 volumes of 95% ethanol. The RNA precipitate was stored in a dessicator at 2° until the time of assay.

The DNA of the supernatant from above (after removal of RNA) was precipitated with an equal volume of 2-ethoxyethanol, and the precipitate was washed twice with an equal volume of 2% sodium acetate in 75% ethanol, once with 95% ethanol, once with absolute ethanol, once with ethanol:ether (1:1), and once with ether. It was then dried under a stream of nitrogen and then dissolved in 1.0 ml of 0.05 M sodium perchlorate. An equal volume of chloroform:isoamyl alcohol (24:1) was added, and the mixture was shaken for 30 min and then centrifuged at 10,000 x g for 10 min to remove protein. The aqueous layer was removed, and DNA was precipitated by the addition of 2 volumes of 95% ethyl alcohol. DNA was dissolved in 1.0 ml of 0.05 M magnesium sulfate in 0.1% sodium acetate (pH 5.5). The DNA was treated with 1.0 ml of RNase (50 µg/ml) at 37° for 30 min. The RNase had been boiled for 15 min prior to use to destroy DNase activity. The DNA was then precipitated with 2 volumes of 95% ethyl alcohol and dissolved in 1.0 ml of 1.0 M sodium perchlorate. Treatment with chloroform:isoamyl alcohol followed and reprecipitation with 2 volumes of 75% ethyl alcohol. The DNA precipitate was stored in a dessicator at 2° until the time of assay.

DNA was assayed fluorometrically by the method of Setaro and Morley (29). RNA was assayed by the orcinol reaction of Schneider (28), and protein, by the method of Lowry et al. (17). Assay of protein precipitate for DNA and RNA showed less than 1% cross-contamination. There was also less than 5% cross-contamination detected between DNA and RNA.

Tissue BP Hydroxylase Activity. Homogenates of various tissues were made in ice-cold 1.15% potassium chloride solution for assay of BP hydroxylase activity by the method of Nebert and Gelboin (19). Liver homogenate was made up as a 10% (wt/v) suspension, and extrahepatic tissues as a 20% suspension. Liver tissue was incubated at 37° for 10 min, and extrahepatic tissue, for 30 min. Values of BP hydroxylase activity represent the amount (in pmol) of the reference standard 3-hydroxybenzo(a)pyrene (provided by Dr. H. V. Gelboin, National Cancer Institute, Bethesda, MD) causing fluorescence equivalent to the total hydroxylated metabolites produced per mg of protein per min of incubation. Fluorescence was measured on an Aminco-Bowman spectrofluorometer (Model 4-8282). Aliquots of the 900 x g supernatant from each tissue homogenate were used for protein determination by the method of Lowry et al. (17).

Tissue Levels of GSH. The method for measuring GSH was adapted from that described by Hisin and Hilf (12) using o-phthalaldehyde. The fluorescence at 420 nm with activation at 350 nm was read on an Aminco-Bowman fluorometer. At pH 8.0, reduced GSH reacts with o-phthalaldehyde. The standard addition method by Boyd et al. (3) was used to correct for quenching of different tissues.

Assay for GSH in split samples of selected organs, freshly prepared and frozen, indicated that the GSH levels remain unchanged after freezing and thawing.

Statistics. Liver weights, BP hydroxylase activity, GSH levels, and BP binding data were compared by using a one-way analysis of variance with the Newman-Keuls method for pairwise comparisons (14, 21). A nonparametric ordered x2 test was used to determine whether there was a difference in the frequency of tumors when groups were compared 2 at a time. The Bonferroni correction was applied to adjust for the multiple comparisons that were made. Probability values of only 0.05 less were considered significant.

RESULTS

Effect of Trifluralin on Body Weight Gain, Liver Weight, and Tissue BP Hydroxylase Activity. Feeding studies with trifluralin were performed to determine the maximum tolerated dose. Trial experiments with various dietary levels of trifluralin in small groups of mice indicated that 1000 or 2000 ppm in the diet for a period of 14 weeks had no significant effect on weight gain (Table 1). Also shown are liver weights as a percentage of body weight, which found to be significantly increased in the animals fed both dietary levels of trifluralin. Gross examination of liver, lung, forestomach, and glandular stomach revealed no tumors in the control animals nor in animals fed the 2 dietary levels of trifluralin for 14 weeks.

Inhibition of BP-induced Forestomach and Lung Tumors by Trifluralin. In our initial study, we investigated the interaction of trifluralin fed at 2 dietary levels for a period of 14 weeks on the number of tumors induced by BP. Gross examination for lung tumors revealed that the percentage of tumors greater than 1 mm was very small (approximately 25% in all groups). A count of the numerous lung tumors, regardless of size, indicated an
TRIFLURALIN INHIBITION OF BENZO(a)PYRENE TUMORIGENESIS

Table 1

<table>
<thead>
<tr>
<th>Diet</th>
<th>Concentration (ppm)</th>
<th>No. of mice</th>
<th>Initial body wt (g)</th>
<th>Final body wt (g)</th>
<th>Weight gain (g)</th>
<th>Liver wt as % of final body wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15</td>
<td>16.0 ± 0.04</td>
<td>23.0 ± 0.04</td>
<td>7.0</td>
<td>783.2 ± 24.2</td>
<td>3.95 ± 0.13</td>
</tr>
<tr>
<td>Trifluralin 1000</td>
<td>15</td>
<td>15.9 ± 0.03</td>
<td>23.1 ± 0.04</td>
<td>7.2</td>
<td>943.3 ± 30.2</td>
<td>4.63 ± 0.10</td>
</tr>
<tr>
<td>Trifluralin 2000</td>
<td>15</td>
<td>16.2 ± 0.03</td>
<td>23.1 ± 0.03</td>
<td>6.9</td>
<td>989.5 ± 21.4</td>
<td>5.00 ± 0.11</td>
</tr>
</tbody>
</table>

- Control diets containing 5% com oil and the experimental diets containing 5% com oil with various levels of trifluralin were fed starting at 9 weeks of age, and after 14 weeks on the diet, mice were sacrificed.
- BP, 3 mg given p.o. twice in 0.25 ml of com oil at 10 and 12 weeks of age.
- *Control diets containing 5% com oil and the experimental diets containing 5% com oil with various levels of trifluralin were fed starting at 9 weeks of age, and after 14 weeks on the diet, mice were sacrificed.
- Mean ± S.E.
- *p < 0.01 versus the control group.

Table 2

| Diet  | Concentration (ppm) | No. of mice | Wt gain (g) | No. of tu-
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung tumors</td>
<td>BP (mg)</td>
<td></td>
<td>mouse</td>
<td>tumors/tumor-bearing mouse</td>
</tr>
<tr>
<td>Forestomach tumors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>3 × 2</td>
<td>37</td>
<td>5.8</td>
<td>97.3</td>
</tr>
<tr>
<td>Trifluralin 1000</td>
<td>3 × 2</td>
<td>26</td>
<td>5.9</td>
<td>96.2</td>
</tr>
<tr>
<td>Trifluralin 2000</td>
<td>3 × 2</td>
<td>27</td>
<td>4.7</td>
<td>74.1</td>
</tr>
</tbody>
</table>

- Control diets containing 5% com oil and the experimental diets containing 5% com oil with various levels of trifluralin were fed starting at 9 weeks of age, and after 14 weeks on the diet, mice were sacrificed.
- BP, 3 mg given p.o. twice in 0.25 ml of com oil at 10 and 12 weeks of age.
- *Weight gain from 9 to 27 weeks of age.
- Tumorigenic index obtained by multiplying the percentage of mice with tumors times the mean number of tumors per tumor-bearing mouse.
- *p < 0.01 versus the control group.

Inhibitory effect by trifluralin on tumor incident. Trifluralin was also found to be protective against BP-induced forestomach tumors. Since only lesions greater than 1 mm are counted as tumors, the experiment was repeated, and the time of feeding trifluralin before sacrifice was lengthened to 18 weeks. The results of this second experiment are presented in Table 2. The longer time of trifluralin feeding resulted in the formation of lung tumors of adequate size (greater than 1 mm) and incidence (97% in controls) following the administration of BP. Both levels (1000 and 2000 ppm) of trifluralin decreased BP-induced tumor formation in the lung when compared with controls. There was a dramatic decrease in the lung tumorigenic index to 30% of control at the higher dietary level of 2000 ppm of trifluralin. Microscopic examination of lung tissue revealed that these tumors were pulmonary adenomas.

Data concerning forestomach tumors are also presented in Table 2. Both levels of trifluralin decreased BP-induced forestomach tumors when compared with controls. In addition, tumor yield in animals fed 2000 ppm trifluralin was significantly lower than in animals fed 1000 ppm. The higher level of trifluralin reduced the tumorigenic index of BP-induced forestomach tumors to 23% of control. Microscopic examination of representative samples of forestomach tumors indicated squamous cell papillomas.

The weight gain of the mice fed 2000 ppm trifluralin was less (4.7 g) than that of the controls (5.8 g). Although this weight difference was not statistically significant after 18 weeks (26 weeks of age) on the groups’ respective diets, there were several time points (at 14, 22, and 24 weeks of age) at which the mean weight of the trifluralin (2000 ppm) group was significantly lower than that of the control group, as illustrated in Chart 1. Therefore, we decided to decrease the maximum dose of trifluralin to 1500 ppm in subsequent experiments.

Effect of Trifluralin on the Binding of BP to Macromolecules of Different Organs. In addition to the initial 14-week feeding study of the effect of trifluralin on the induction of tumors by BP, we ran a separate group of animals fed trifluralin to determine its effect on the binding of p.o.-administered BP to DNA, RNA, and protein in liver and in lung. The same protocol for inducing BP tumors was followed...Mice were started on the trifluralin or control diets at 9 weeks of age, and BP (3 mg) was given p.o. at 10 weeks of age. At 12 weeks of age, 150 mg of BP plus 15 mCi of [3H]BP per kg (in corn oil) were given p.o., and mice were sacrificed 24 hr later. Based on animal weight, the second dose of BP (150 mg/kg) is equivalent to approximately 3 mg of the single p.o. dose used in our tumor studies. Table 3 shows the results in ng of [3H]BP bound per mg of macromolecule. Feeding of trifluralin at 1000 and 2000 ppm significantly reduced the binding of [3H]BP to DNA in the liver; at the higher dose of trifluralin, there was also a decreased binding to protein. In the lung, significant inhibition of binding was found only for DNA in animals treated with 2000 ppm of trifluralin. Binding in the forestomach after trifluralin feeding was not determined due to the large number of animals that would be required for pooling of this small tissue.

Effect of Trifluralin on Tissue BP Hydroxylase Activity and GSH Content. We determined the effect of trifluralin on tissue BP hydroxylase activity and GSH content at the time of exposure to the first dose of BP required for tumor induction. For these experiments, we fed trifluralin in the diet for a period of only 2 weeks, starting at 9 weeks of age. The level of trifluralin in the diet was reduced to 1500 ppm because our previous experi-
TRIFLURALIN INHIBITION OF BENZO(a)PYRENE TUMORIGENESIS

Chart 1. Effect of trifluralin fed in the diet and p.o. administered BP on weight gain in female A/J mice. Points, biweekly determinations of mean weights; bars, S.E. *, significance level (p < 0.05 from control).

Table 3

Effect of trifluralin fed in the diet on macromolecular binding of [3H]BP to the liver and lung of female A/J mice

<table>
<thead>
<tr>
<th>Diet</th>
<th>Concentration (ppm)</th>
<th>Liver (ng BP bound/mg)</th>
<th>Lung (ng BP bound/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DNA</td>
<td>RNA</td>
<td>Protein</td>
</tr>
<tr>
<td>Control</td>
<td>18.4 ± 1.9d</td>
<td>12.3 ± 2.0</td>
<td>62.9 ± 6.1</td>
</tr>
<tr>
<td>Trifluralin</td>
<td>9.5 ± 0.6d</td>
<td>7.7 ± 2.7</td>
<td>51.8 ± 8.7</td>
</tr>
<tr>
<td>Trifluralin</td>
<td>8.9 ± 0.1d</td>
<td>5.4 ± 2.4</td>
<td>34.1 ± 5.7</td>
</tr>
</tbody>
</table>

a Control diet containing 5% corn oil and the experimental diets containing 5% corn oil with various levels of trifluralin were fed starting at 9 weeks of age, and after 3 weeks on the diet, mice were sacrificed.

BP, 3 mg given p.o. in 0.25 ml of corn oil at 10 weeks of age, and BP, 150 mg, plus [3H]BP, 15 mCi/kg given p.o. in corn oil (1% body weight), at 12 weeks of age. Twenty-four hr after the last dose of BP, mice were sacrificed, and their livers and lungs were removed and frozen in liquid nitrogen. Each determination, for liver, was performed with tissues pooled from 2 mice, and for lung, tissues from 7 mice were used. These values assume that all detected radioactivity represents BP adducts.

b Mean ± S.E. of 3 to 6 determinations.

c p < 0.05 versus the control group.

d p < 0.01 versus the control group.

e p < 0.01 versus the 1000-ppm trifluralin group.

Table 4

Effect of trifluralin fed in the diet on tissue BP hydroxylase activity in female A/J mice

<table>
<thead>
<tr>
<th>Diet</th>
<th>Concentration (ppm)</th>
<th>Liver BP hydroxylase activity</th>
<th>Lung BP hydroxylase activity</th>
<th>Foregut stomach BP hydroxylase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Protein (pmol/mg protein/min)</td>
<td>Protein (pmol/mg protein/min)</td>
<td>Protein (pmol/mg protein/min)</td>
</tr>
<tr>
<td>Control</td>
<td>29.5 ± 3.5d</td>
<td>0.063 ± 0.006</td>
<td>0.034 ± 0.001</td>
<td></td>
</tr>
<tr>
<td>Trifluralin</td>
<td>30.3 ± 1.9</td>
<td>0.089 ± 0.005</td>
<td>0.031 ± 0.005</td>
<td></td>
</tr>
<tr>
<td>Trifluralin</td>
<td>40.7 ± 6.2</td>
<td>0.063 ± 0.004d,e</td>
<td>0.03 ± 0.003</td>
<td></td>
</tr>
</tbody>
</table>

a Control diets containing 5% corn oil and the experimental diets containing 5% corn oil with various levels of trifluralin were fed starting at 9 weeks of age, and after 2 weeks on the diet, mice were sacrificed.

b Expressed as pmol of 3-hydroxybenzo(a)pyrene per mg of protein per min.

c Mean ± S.E. of 4 to 8 determinations. Each determination was performed with tissues pooled from 2 mice, except foregut stomach, in which pooled tissues from 4 mice were used.

d p < 0.01 versus the control group.

e p < 0.05 versus the 1000-ppm trifluralin group.

ments indicated that higher levels of trifluralin (2000 ppm) may suppress the rate of weight gain (Chart 1). Table 4 presents data revealing that BP hydroxylase activity in trifluralin-treated animals was higher than that in controls in liver, lung, and glandular stomach, but the difference was not significant. However, in the forestomach, we found a significant decrease in BP hydroxylase activity in animals fed 1500 ppm of trifluralin.

The effect of feeding trifluralin for 2 weeks on GSH concentrations in several organs is shown in Table 5. In the lung, the GSH content was significantly increased above the control levels by dietary feeding of 1000 and 1500 ppm of trifluralin. In the forestomach, there was a significant increase in GSH content.
The diet of mice starting at 9 weeks of age and up to 1.5 days before the last dose of BP. At this time interval, there was a maximum increase in the tumorigenic index which was decreased by 40% when trifluralin feeding was started at the 1-day interval after the last dose of BP. At this time, the formation of BP-induced tumors was determined by feeding the diet as early as 1 day following the last dose of BP. The time of maximum protection, as determined by the percentage of change in the tumorigenic index, appears to occur when trifluralin is started in the diet as early as 1 day following the last dose of BP. The effect of trifluralin on the formation of BP-induced tumors was determined by feeding trifluralin (1500 ppm) in the diet starting simultaneously with the first dose of BP; in a second group, trifluralin was fed up to 1.5 days before the second dose of BP. Animals fed trifluralin were then switched to the control diet containing 5% corn oil and sacrificed at 20 and 23 weeks of age for tumor analysis. When fed by this protocol, trifluralin did not affect the incidence of either lung or forestomach tumors induced by BP (data not presented).

**DISCUSSION**

Our data show that, when trifluralin was fed in the diet of the A/J strain of mouse both before and after the p.o. administration of BP, this herbicide significantly inhibited the induction of lung and forestomach tumors. Many possible mechanisms may explain this protection against BP-induced tumors. Some antitumorogenic agents, termed "blocking" agents, prevent carcinogens from reaching or reacting with macromolecules, such as DNA, in target organs. Agents that protect against tumorigenesis when given subsequent to the carcinogen have been termed "suppressive" agents. The time relation between the feeding of a test compound and the administration of the carcinogen is important in revealing a "blocking" or a "suppressing" effect in tumorigenesis (for review, see Ref. 41).

**Table 5**

<table>
<thead>
<tr>
<th>Diet</th>
<th>Concentration (ppm)</th>
<th>Liver</th>
<th>Lung</th>
<th>Foregastro</th>
<th>Glandular stomach</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.82 ± 0.74</td>
<td>2.38 ± 0.22</td>
<td>5.96 ± 0.45</td>
<td>9.66 ± 0.38</td>
<td></td>
</tr>
<tr>
<td>Trifluralin 1000</td>
<td>8.89 ± 1.47</td>
<td>4.61 ± 0.30</td>
<td>7.81 ± 0.22</td>
<td>10.80 ± 1.10</td>
<td></td>
</tr>
<tr>
<td>Trifluralin 1500</td>
<td>8.09 ± 0.76</td>
<td>4.29 ± 0.66</td>
<td>10.74 ± 1.37</td>
<td>9.60 ± 0.42</td>
<td></td>
</tr>
</tbody>
</table>

*Control diets containing 5% corn oil and the experimental diets containing 5% corn oil with various levels of trifluralin were started at 9 weeks of age, and after 2 weeks on the diet, mice were sacrificed. p < 0.01 versus the control group. p < 0.05 versus the control group.

Only at the level of 1500 ppm. There was no significant change in the GSH levels in either the liver or glandular stomach after feeding trifluralin.

**Inhibition by Trifluralin of BP-induced Tumors When Fed following the Administration of BP.** The effect of trifluralin on the formation of BP-induced tumors was determined by feeding trifluralin (1500 ppm) in the diet starting simultaneously with the last dose of BP, as well as 1, 4, and 7 days after the last dose. Data in Table 6 show a significant reduction of lung tumors at all time intervals of feeding trifluralin after BP. The time of maximum protection, as determined by the percentage of change in the tumorigenic index, appears to occur when trifluralin is started in the diet as early as 1 day following the last dose of BP. At this time interval, the tumorigenic index is decreased by 69%. Data for the forestomach show that significant protection occurs only when trifluralin feeding was started at the 1-day interval after the last dose of BP. At this time interval, there was a maximum change in the tumorigenic index which was decreased by 40%.

An experiment was designed to determine if the protective effect of trifluralin against BP-induced tumors would develop after short-term feeding (5.5 or 19.5 days) of trifluralin prior to the administration of BP. Trifluralin, at 1500 ppm, was fed in the diet of mice starting at 9 weeks of age and up to 1.5 days before the first dose of BP; in a second group, trifluralin was fed up to 1.5 days before the second dose of BP. Animals fed trifluralin were then switched to the control diet containing 5% corn oil and sacrificed at 20 and 23 weeks of age for tumor analysis. When fed by this protocol, trifluralin did not affect the incidence of either lung or forestomach tumors induced by BP (data not presented).
TRIFLURALIN INHIBITION OF BENZO(a)PYRENE TUMORIGENESIS

Induction of cytochrome P-450-dependent monooxygenase enzymes in several tissues has been postulated as a mechanism for the antitumorogenic effects of a wide variety of compounds such as flavones, coumarins, phenothiazines, insecticides (dichlorodiphenyltrichloroethane and chlor dane), and phenolic antioxidants (24, 38, 40, 42). We investigated the "blocking" effect of trifluralin fed in the diet on tissue BP hydroxylase activity (a cytochrome P-450-dependent monooxygenase), an enzyme system that is responsible for both the activation and inactivation of BP. After the feeding of trifluralin (1000 or 1500 ppm) for a period of 2 weeks, there were no significant changes in liver, lung, or glandular stomach enzyme activity. However, forestomach BP hydroxylase activity was significantly inhibited by 1500 ppm of trifluralin (Table 4). The inhibition of forestomach BP hydroxylase may be due to the fact that trifluralin fed in the diet is metabolized by microsomal cytochrome P-450 enzymes present in the forestomach (20). Thus, an excess of substrate (e.g., trifluralin) in contact with forestomach enzyme may result in decreased activity. The absence of change in lung enzyme may be due to the short feeding time (2 weeks) of trifluralin. From these findings, it appears that changes in tissue BP hydroxylase activity by short-term feeding of trifluralin cannot account for the changes observed in BP-induced tumors that subsequently develop in the respective target organs following continued feeding of trifluralin for a duration of 12 weeks.

The "blocking" effect of trifluralin against BP-induced tumors may be mediated by changes in tissue GSH content, which can detoxify reactive intermediate epoxides of BP (4, 25, 26). No changes in the level of GSH in nontarget organs (liver and glandular stomach) were observed after short-term feeding of trifluralin. However, in target organs (forestomach and lung), we found a significant increase in GSH levels when trifluralin was fed for only 2 weeks (Table 5). An increase in tissue GSH would conjugate reactive intermediate epoxides of BP, resulting in less binding to DNA, and thus provide protection against BP tumorogenicity. The antioxidant, butylated hydroxyanisole, has been shown by others to protect forestomach and lung against BP-induced tumors in mice. This protection was attributed by the investigators to increased levels of GSH (36, 37, 39). In our procedure for BP tumor induction, we observed a significant decrease of BP binding to liver DNA and protein after feeding trifluralin for 3 weeks. There was also a significant decrease in BP binding to DNA of lung after the feeding of 2000 ppm of trifluralin (Table 3). Consistent with our results, Anderson et al. (1) showed a decrease in BP binding to DNA in the lung following treatment with butylated hydroxyanisole, shown previously to reduce BP-induced pulmonary tumors (37). In the lung, our data showed that trifluralin decreased the binding of BP metabolites to macromolecules, increased GSH levels, and protected against BP tumorogenesis.

In other experiments, we found that trifluralin protected against BP-induced tumors in the lung and forestomach when started in the diet 1 week before the first p.o. dose of BP and continued for a duration of 14 weeks (data not presented) or 18 weeks (Table 2). Since trifluralin was fed not only before but also subsequent to the administration of BP, it appears from the data that the protective effects obtained could be due to a "blocking" effect and/or a "suppressing" effect. To further elucidate the mechanisms involved in this protection, we fed trifluralin up to 1.5 days before the first or 1.5 days before the second dose of BP. In these experiments (data not presented), there was no protection by trifluralin against the incidence of lung or forestomach tumors. However, when trifluralin feeding was started simultaneously with the second dose of BP and as late as 7 days after, there was significant protection at all time intervals against lung tumors (Table 6). Significant protection against forestomach tumors was found only when trifluralin feeding was started 1 day after the second p.o. dose of BP (Table 6). Continuous trifluralin feeding produced the same degree of protection against lung tumors whether it was started before or 1 day after BP administration. This can be seen by the change in the tumorogenic index which was found to decrease from 100% in the control groups to approximately 30% in the trifluralin-treated groups (Tables 2 and 6). The mechanisms responsible for the "suppressive" effect by chemical agents when administered subsequent to the carcinogen could involve changes in the rate of mitosis and/or DNA repair. It has been reported that actinomycin D protects against skin tumors when given after DMBA by inhibiting mitosis (9, 10). Caffeine when given after urethane also has been reported to protect against pulmonary tumors by inhibiting error-prone postreplication repair of DNA (22).

From our results, it appears that the "suppressing" effect of trifluralin is just as effective as its "blocking" effect in protecting against BP-induced tumors. Fewer mechanisms are involved in the "suppressive" effect than in the "blocking" effect of antitumorogenic agents. Therefore, to understand the "suppressive" effect of trifluralin, further studies should focus on the mechanisms involved when this herbicide is fed after the administration of BP.

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REFERENCES

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Protective Effects of Trifluralin on Benzo(a)pyrene-induced Tumors in A/J Mice

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