Letter to the Editor

Induction of Rat Mammary Adenomas with the Respiratory Inhibitory Rotenone

We have tried the well-characterized respiratory inhibitor rotenone (1) and found it to be consistently tumorigenic in the albino rat. This finding, together with the mitochondrial characteristics observed in the rotenone-induced tumors, is reported here. Rotenone tumors represent a class of tumors produced by small amounts of a chemical compound with a defined biochemical action. Furthermore, the tumorigenicity of rotenone could be of ecological significance, because rotenone is at present used as an insecticide (4).

Four series of 10 female albino rats (inbred strain with a low natural mammary tumor incidence, 0.5 tumors/1000 rats/year) weighing 100 ± 1 g each (35 ± 2 days old) were given i.p. injections of rotenone (K & K Laboratories, Inc., Plainview, N. Y.) (1.7 µg/g rat weight, dissolved in 0.1 ml of sunflower oil) daily for 42 days. The total dose administered was 9.1 ± 1.6 mg of rotenone per rat. Eighty% of the rats survived the treatment in the 1st series; 90% survived in the other 3 series. The deaths were due to acute peritonitis. Four other series of 10 control rats were given daily injections of 0.1 ml of the solvent for the same period of time.

In the 1st series of 10 rotenone-treated rats (treatment started in February 1971), we observed an incidence of 100% mammary tumors that appeared from 6 to 11 months after the end of treatment. In none of the controls of this series was any tumor found upon macroscopic and microscopic examination, performed 19 months after the treatment. In the other 3 series of 10 rotenone-treated rats (treatment started in June 1972), an incidence of 60% mammary tumors has been observed so far, 10 months after the end of the treatment, while none of the controls has shown any tumor.

The 8 tumors obtained from the 1st series were extirpated for morphological studies and transplantation. Five of the tumors were used for further biochemical study of their isolated mitochondria (Table 1). Seven of these tumors were histologically diagnosed as mammary adenomas with accentuated interstitial fibrosis and showed localized areas with adenocarcinomatous transformation; 1 was diagnosed as a differentiated adenocarcinoma. All tumors were encapsulated and did not show metastasis. Macroscopic and microscopic examination of the animals bearing tumors did not show any liver damage or changes in the endocrine organs. In spite of their histological benignancy, some of the tumors were transplantable in normal young or older rats (4 to 5 successful transplants out of 30 trials). Rotenone primary or transplanted tumors are slow-growing tumors, and generally 7 to 12 months are needed for full development of the tumor after initial detection.

Electron microscopy of the tumor tissue showed a gradation of mitochondrial lesions ranging from normal mitochondria with scarce, short, and anarchically distributed cristae to mitochondria with partially disintegrated inner and outer membranes, devoid of cristae and with a fuzzy matrix. The pellets of mitochondria isolated from the tumors contained all kinds of mitochondrial material corresponding to the different aspects of mitochondria in situ: well-preserved mitochondria in a swelled or contracted state, mitochondrial vesicles with a single membrane, and mitochondrial fragments. The proportion of apparently intact mitochondria varied between 15 and 25% of the total mitochondrial material, depending on the preparation. The isolation of such a poor mitochondrial preparation from rotenone tumors could be explained on the basis of the observed structural damage of mitochondria within the tissue. The differential spectra (reduced minus oxidized) of the mitochondrial preparations always showed the presence of cytochromes a, b, and c (M. Gosalvez and L. Salganicoff, manuscript in preparation).

Fig. 1A shows a typical rotenone primary tumor (RPT-6), observed in a rat immediately before it was killed. Also shown are the histological appearance of the tumor (Fig. 1B) and details of its mitochondria in situ obtained by electron microscopy (Fig. 1C).

Table 1 shows the respiration with NADH, NADH-linked substrates, and succinate, the respiratory control, and the P/O ratio of mitochondria isolated from rotenone-induced tumors, in comparison with the same parameters obtained with mitochondria isolated from a spontaneous mammary adenoma that appeared in an albino rat from our colony. Data on mitochondria of normal mammary tissue isolated by the same technique are also shown. Included in the table are the amount of mitochondrial protein/g of tissue and the aerobic and anaerobic glycolysis for 3 of the tumors. All rotenone tumors, except RPT-8, which was extirpated only 3 months after initial detection, lacked respiration with glutamate and malate in spite of the presence of apparently intact mitochondria in the mitochondrial preparations. Similar results were obtained with other NADH-linked substrates and in the presence of 2 mM NAD (not shown).

1 The work reported in this paper was supported by the Instituto Nacional de Previsión, Grant 12-079-72 (M. G.), and by an A.E.C.C. grant (J. M.).

2 It can be estimated that during the past 40 years the world consumption of rotenone as an insecticide has been from 10,000 to 20,000 kg/year (data obtained from the Section of Agricultural Plagues, Ministry of Agriculture, Madrid, Spain).

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Table 1
Mitochondrial properties and glycolysis of rat mammary adenomas induced by rotenone

Rotenone mammary tumors 4, 5, 6, and 7 were extirpated 6 months after initial detection, and their weights ranged from 40 to 63 g. RPT-8 was extirpated 3 months after initial detection, and its weight was 20.2 g. Mitochondria were isolated by the method of Nelson and Butow (6) for the mammary gland. Respiration was determined in a sensitive Clark-type oxygen electrode with electronics designed at the Johnson Research Foundation (University of Pennsylvania) and is expressed in nanomoles of oxygen per min per mg of mitochondrial protein. The assay medium for respiration was 0.25 M sucrose, 20 mM KCl, 7 mM MgCl2, 10 mM Tris-HCl, and 5 mM P (pH 7.4, 22°). The substrates were added to give the indicated final concentrations: glutamate, 5 mM; malate, 5 mM; succinate, 6 mM; ascorbate, 3 mM; tetramethyl-p-phenylenediamine, 0.2 mM. Phosphorylation was determined by assaying glucose 6-phosphate 30 min after the addition of hexokinase, 1.4 units/ml; glucose, 20 mM; and ATP, 0.5 mM (7). Respiratory control was determined by the direct addition of ADP (0.3 mM) (2). Glycolysis was determined in slices, as described previously (8), and is expressed in mmoles of lactate per hr per mg of dry weight. Mitochondrial protein is expressed in mg and was determined by the biuret reaction.

<table>
<thead>
<tr>
<th>Tumors</th>
<th>Mitochondrial protein/g tissue</th>
<th>Respiration with glutamate and malate</th>
<th>Respiration with succinate</th>
<th>Respiration with ascorbate plus tetramethyl-p-phenylenediamine</th>
<th>Glycolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Without ADP</td>
<td>With ADP</td>
<td>Respiratory control</td>
<td>P;O</td>
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<tr>
<td>RPT-4</td>
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<td>0.0</td>
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<tr>
<td>RPT-5</td>
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<td>0.0</td>
<td>0.0</td>
<td>5.6</td>
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<tr>
<td>RPT-6</td>
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<td>0.0</td>
<td>0.0</td>
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</tr>
<tr>
<td>RPT-7</td>
<td>2.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>4.1</td>
</tr>
<tr>
<td>RPT-8</td>
<td>1.3</td>
<td>0.0</td>
<td>1.5</td>
<td>1.5</td>
<td>3.0</td>
</tr>
<tr>
<td>Spontaneous adenoma</td>
<td>1.3</td>
<td>2.0</td>
<td>2.0</td>
<td>4.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Normal mammary tissue</td>
<td>2.5</td>
<td>0.0</td>
<td>4.0</td>
<td>24</td>
<td>6</td>
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</table>

* N.D., not done.
phosphorylation and respiratory control were undetectable with all substrates in all rotenone tumors. Aerobic and anaerobic glycolysis was of the same order as that found in malignant mammary tumors (8). Compared with the tumors induced with rotenone, the spontaneous mammary adenoma showed respiration with all substrates, low respiratory control, almost normal oxidative phosphorylation, and some respiration with NADH.

From the results obtained in the 1st series of tumors, rotenone tumors seem to be characterized by mammary localization, histological benignancy, a structural mitochondrial deletion accompanied by poor mitochondrial functions, and a glycolysis rate similar to that found in malignant mammary tumors. In the other 3 series of tumors, attempts are being made to study the progression of these characteristics with tumor development. The age of the animals is not essential in rotenone tumorigenicity. Various series of older rats treated with rotenone started to show a high incidence of mammary tumors.

Several possible explanations exist for the tumorigenicity of rotenone. Rotenone may act as a hormone analog or may indirectly affect the secretion or detoxification of steroid hormones. Against this possibility stands the fact that the age of the animal is not critical and that no histological change is observed in the liver or endocrine organs of the animals bearing tumors. On the other hand, commercial rotenone could include as an impurity some active known or unknown carcinogen. Chromatographic analysis (3) of the batch of rotenone that we used showed less than 1% impurities, composed mostly of rotenoids (epirotenone, rotenolones, and dehydrorotenol) and traces of a spiro compound. It can be estimated that our rats received in total no more than 0.1 mg of rotenoids, which makes it very unlikely that any of the rotenoids could be the carcinogen. Another possible explanation for the production of tumors with rotenone could be the selection of respiratory mutant cells which would be responsible for the tumoral transformation. The mitochondrial deficiencies of rotenone tumors seem to be interesting in this respect. Recently, it was reported (5) that, in mutant yeast cells, resistance to rotenone could be the selection of respiratory mutant cells which would be responsible for the tumoral transformation. The mitochondrial deficiencies of rotenone tumors seem to be interesting in this respect. Recently, it was reported (5) that, in mutant yeast cells, resistance to rotenone is controlled by the nuclear genome.

Fig. 1. A, a typical tumor induced by rotenone (RPT-6), localized in a thoracic mammary gland, as seen in a rat before it was killed 13 months after the end of rotenone treatment. B, histological appearance of tumor RPT-6 (mammary fibroadenoma). Van Gieson, × 340. C, ultrafine section of tumor RPT-6 detailing representative aspects of its mitochondria. A gradation of mitochondrial lesions is observed. Partially disintegrated inner and outer membranes are visible in the mitochondrion on the left. Disintegrated cristae and matrix are apparent in the mitochondrion in the center. The mitochondrion on the right is nearly normal. Glutaraldehyde-osmium, × 92,160.

Despite the presence of broken mitochondria and mitochondrial vesicles in the preparations, all rotenone tumors failed to show respiration with NADH, and 2 tumors did not show respiration with succinate. A reasonably high level of cytochrome oxidase was present in the mitochondria of all rotenone tumors, as indicated by the respiration with ascorbate and tetra-p-phenylenediamine. Oxidative phosphorylation and respiratory control were undetectable with all substrates in all rotenone tumors. Aerobic and anaerobic glycolysis was of the same order as that found in malignant mammary tumors (8). Compared with the tumors induced with rotenone, the spontaneous mammary adenoma showed respiration with all substrates, low respiratory control, almost normal oxidative phosphorylation, and some respiration with NADH.

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REFERENCES

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