Production of Tumors in Rats by 2-Aminofluorene and 2-Acetylaminofluorene

Failure of Liver Extract and of Dietary Protein Level to Influence Liver Tumor Production

Paul N. Harris, M.D.

(From the Lilly Research Laboratories, Eli Lilly and Company, Indianapolis 6, Indiana)

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The carcinogenic property of 2-acetylaminofluorene was first described by Wilson and his associates (9) in 1941. Of 39 rats that received the compound in the food at a level of 0.031 to 0.125 per cent for 95 or more days, 19 developed malignant tumors, and 8 animals had multiple tumors. These included 10 bladder carcinomas; 8 epidermoid carcinomas of the side of the face; 3 mammary carcinomas; 3 hepatic carcinomas; 1 carcinoma arising in each of the following sites: ureter, kidney pelvis, colon, pancreas, and lung; and 1 rhabdomyosarcoma of the thigh. The rats were derived from the Slonaker strain, and the majority were female. One-half gram of the compound was injected subcutaneously into 5 male rats, and no tumors had developed 14 months later.

In 1944 Bielschowsky (2), using rats derived from the Wistar strain, and feeding at the level of 4 mgm. of the carcinogen per day, found malignant tumors in 93 of 104 animals. Some animals had multiple tumors. Fifty-five rats had liver carcinoma, 27 had mammary carcinoma, 16 had carcinoma of the acoustic duct, 5 had carcinoma of the intestine, 1 had carcinoma of the skin, and 1 had a tumor of the uterus. In addition, 4 animals developed leukemia, and 11 had pulmonary adenomas. In male rats liver tumors were most numerous, and mammary carcinoma was relatively uncommon, but in female rats mammary carcinoma was twice as common as liver carcinoma.

Bielschowsky also found the compound to be innocuous when injected subcutaneously, and surmising that the subcutaneous tissues could not split off the acetyl group, applied a 4 per cent solution of 2-aminofluorene in acetone thrice weekly to the skin of 5 male rats for a period of 210 days. After 280 days, liver tumors were found in all 5 male rats for a period of 210 days. After 280 days, liver tumors were found in all 5 rats, and 1 rat also had an acoustic duct tumor.

By simultaneous feeding of 2-acetylaminofluorene and allylthiourea, Bielschowsky (3) produced benign and malignant thyroid tumors, but by successive feeding of these compounds (4) thyroid tumors were not produced. Carcinomas of the small intestine, liver, mammary gland, and acoustic duct developed in some of the rats in both experiments.

Armstrong and Bonser (1) administered 0.2 cc. of a 1.5 per cent suspension of 2-acetylaminofluorene in olive oil by gavage thrice weekly to CBA mice, and of 10 that survived 32 to 65 weeks of treatment, 8 developed tumors. Five had bladder tumors (4 were malignant); 5 had liver tumors (1 was malignant); and 2 had uterine tumors.

Lopez (7) reported development of a glioblastoma of the cerebrum in 1 of 12 rats that had been fed a diet containing 0.05 per cent of 2-acetylaminofluorene. At the time his article was written, 7 rats were still living and 4 had developed tumors. In addition to the brain tumor, there were 2 liver tumors, 2 mammary tumors, and 3 acoustic duct tumors.

Heiman and Meisel (6) administered 2-acetylamino-fluorene to 59 Wistar rats by introducing a needle into the pharynx and expelling 1 cc. of peanut oil containing 10 mgm. of the carcinogen. At first, treatment was given every other day, but later the dose was doubled and given every third day. Twenty-two rats developed nodular swellings in one or both submaxillary regions. Subsequently, enlarged submaxillary glands and oil-containing cysts were found, the latter having resulted from perforation of the pharynx. Only 12 animals developed tumors. These included 2 adenocarcinomas and 3 adenomas of the submaxillary glands (the latter appearing as isolated areas of hyperplastic ducts and acini), 2 parathyroid adenomas, 2 mammary carcinomas, 1 thyroid adenoma, 1 liver adenoma, and 1 sarcoma of the neck. Twenty-four rats received aromatic amino acids over a period of 45 days and none developed tumors or hepatic cirrhosis. The authors attributed development of tumors in the vicinity of the oil cysts to local action of 2-acetylaminofluorene.
In view of the high incidence of liver tumors in Bielschowsky's rats, it was proposed to ascertain whether or not some of the dietary factors that are known to influence the carcinogenic action of p-dimethylaminoazobenzene would have the same effect upon carcinogenesis by 2-acetylaminofluorene and 2-aminofluorene. Since the available amount of these 2 compounds was small, only a few experiments could be carried out.

**METHODS**

Our rats were obtained from a local breeder and were descended from the Wistar strain. The carcinogens were obtained from Eastman Kodak Company. The 2-acetylaminofluorene was incorporated in the diet at a level of 0.04 per cent and the rats were fed *ad libitum*. The 2-aminofluorene was dissolved in benzene in a concentration of 4 per cent and was painted on the abdominal skin for 147 days (from October 20, 1944 until March 16, 1945). Applications were made twice weekly for 3 months and 5 times weekly for 2 months, with a total of 62 treatments.

The rats that received the acetyl derivative were divided into three groups, each of which was placed on a different synthetic diet (diets 1, 2, and 3). These three diets were chosen because they were very similar to diets that we (5) have found to influence profoundly p-dimethylaminoazobenzene carcinogenesis (see Fig. 1, curves 23, 25; and 26). The composition of these diets is given in Table I. Diet 1 is comparable to diet 23, but contains 3 per cent less fat. Diets 2 and 3 are comparable to diets 25 and 26, respectively, but differ in containing 3 per cent more casein and greater amounts of some of the members of the vitamin B group. These differences were introduced with the idea that use of basal diet C with 2-acetylaminofluorene might be accompanied by a high mortality rate, and that slight increase in certain factors, which experience had shown would have little effect upon p-dimethylaminoazobenzene carcinogenesis, might also have little effect upon 2-acetylaminofluorene carcinogenesis, but appreciable capacity to diminish the mortality rate. It now appears probable that this precaution was unnecessary.

The rats that were painted with the solution of 2-aminofluorene were divided into two groups. One group was fed with basal diet B (diet 4), and the other was fed with our regular colony diet (diet 5), an excellent ration that promotes rapid growth.

After 4 months of treatment, the rats were examined weekly for tumors. The presence of liver tumors was determined by palpation of the abdomen. This procedure was not entirely satisfactory, since the fluorene derivatives usually caused considerable enlargement of the liver before tumors developed. Even when tumors appeared, they often protruded only slightly above the surface of the surrounding tissue and could not be palpated readily. As soon as it seemed certain that a liver tumor was present, the rat was killed and its viscera...
were examined. A few animals were killed because of mammary or acoustic duct tumors that had reached a size or condition deleterious to the animal's health, and in some of these liver tumors were found. Consequently, in some animals tumors were found soon after they had developed, and in others tumors had been present for several weeks before they were discovered.

RESULTS

It will be seen by reference to Fig 1, which shows percentage incidence of liver tumors plotted against time, that variation in casein and riboflavin content of the diet had no effect upon production of liver tumors by 2-acetylaminofluorene (curves 1 and 2). Liver extract appeared to retard tumor development slightly (curve 3), but the effect is of doubtful significance. For all diets in Fig. 1, death of a tumor-free animal or of 1 that had other than a liver tumor is indicated by a short line perpendicular to the curve. For comparison, the effects of liver extract (curve 26), and of increased amounts of riboflavin and casein (curve 23), as contrasted with a diet low in riboflavin and casein (curve 25), upon \(\beta\)-dimethylaminoazobenzene carcinogenesis are shown in the same figure. Diet 26 greatly retards development of liver tumors, and diet 23 has a similar, but weaker effect.

If female rats are excluded from the calculations as a means of minimizing the complications introduced by mammary tumors, the slope of curves 1, 2, and 3 is a little more steep and the ultimate liver tumor percentages are 100, 100, and 85, respectively, and the curve for diet 2 never crosses that of diet 1, but always runs a little ahead of it. The curves for the three diets thus have the same relative order as do those of the three corresponding \(\beta\)-dimethylaminoazobenzene diets, but the difference in the rate of tumor formation for the 2-acetylaminofluorene diets is too slight to be significant.

Although variation in the diet had no appreciable effect upon the tumor incidence, comparison of the number of animals in each group at the beginning of the experiment with the effective number (see Table II) will show that it did have a decided effect upon the mortality rate during the latent period. During this time the mortality rate on diet 1 was 28 per cent, on diet 2, 43 per cent, and on diet 3, 10 per cent. The number and location of tumors obtained are also shown in Table II.

Table II: Tumor Incidence According to Sex and Diet

<table>
<thead>
<tr>
<th>Diet number</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>Total</th>
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<tbody>
<tr>
<td>Sex</td>
<td>M</td>
<td>F</td>
<td>M</td>
<td>F</td>
<td>M</td>
<td>F</td>
</tr>
<tr>
<td>No. rats in each group</td>
<td>20</td>
<td>5</td>
<td>20</td>
<td>10</td>
<td>15</td>
<td>5</td>
</tr>
<tr>
<td>Effective no.</td>
<td>13</td>
<td>5</td>
<td>9</td>
<td>8</td>
<td>14</td>
<td>4</td>
</tr>
<tr>
<td>No. that developed tumor</td>
<td>13</td>
<td>4</td>
<td>9</td>
<td>7</td>
<td>12</td>
<td>4</td>
</tr>
<tr>
<td>Total with liver tumor</td>
<td>13</td>
<td>4</td>
<td>9</td>
<td>3</td>
<td>11</td>
<td>3</td>
</tr>
<tr>
<td>Liver tumor only</td>
<td>10</td>
<td>1</td>
<td>6</td>
<td>---</td>
<td>7</td>
<td>---</td>
</tr>
<tr>
<td>Acoustic duct tumor</td>
<td>2</td>
<td>---</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Mammary tumor</td>
<td>---</td>
<td>2</td>
<td>---</td>
<td>5</td>
<td>---</td>
<td>3</td>
</tr>
<tr>
<td>Bladder tumor</td>
<td>---</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>---</td>
<td>1</td>
</tr>
<tr>
<td>Pulmonary adenoma</td>
<td>---</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>---</td>
<td>1</td>
</tr>
<tr>
<td>Other tumors*</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>---</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

* Including 1 adenocarcinoma of ileum, 1 epidermoid carcinoma of lung, 3 papillary epidermoid carcinomas of buccal mucosa, 1 papilloma of 8p, 1 papilloma of forestomach, and 1 fibroma of flank. Two rats had a small cavernous hemangioma of liver.

DESCRIPTION OF FIGURES 2 TO 5

Fig. 2—Pulmonary adenoma. Mag. × 140.  
Fig. 4—Epidermoid carcinoma of acoustic duct. Mag. × 140.  
Fig. 3—Epidermoid carcinoma of bladder. Mag. × 140.  
Fig. 5—Mammary carcinoma. Mag. × 140.
Figs. 6-9
The number of rats that developed liver tumors following painting with 2-amino fluorene was too small to justify much discussion of the effect of diet, but certain facts are of interest. Four rats on diet 4 died of pneumonia 123 to 137 days after treatment was begun, and 2 rats on diet 5 died of pneumonia 157 and 171 days after painting was begun. It was thought that the stock diet (no. 5) might offer protection against tumor development, yet the first liver tumor was found at 137 days in a rat getting this diet, and 2 more rats in this group had developed liver tumors by the time the first liver tumor was found in the other group (diet 4). However, when liver tumors began to appear on diet 4 they did so rapidly (the latent period ranged from 242 to 322 days), and the slope of the curve of tumor incidence made an angle of 60° with the abscissa. The latent period on diet 5 ranged from 137 to 405 days, and the slope of the curve of tumor incidence made an angle of 30° with the abscissa. Hence, there is no clear-cut evidence that diet had any effect upon development of liver tumors.

The acoustic duct tumors were all epidermoid carcinomas. The majority were of low grade malignancy and were sharply circumscribed. They consisted of large papillary masses of squamous epithelial cells with a delicate fibrous tissue core. The surface of the masses was covered with a thick layer of keratinized cells. Fig. 4 presents an example. Two of the tumors showed much anaplasia and were highly invasive; in several of the better differentiated tumors, there was beginning invasion of the surrounding tissues. Many of these tumors were infected by the time they were removed for section. They had a sour odor and were filled with flaky gray material. Two rats had bilateral tumors.

One mammary tumor was of intracystic papillary type and appeared benign. The other mammary tumors were, with one exception, adenocarcinomas of various grades of malignancy (see Fig. 5). The exception was an epidermoid carcinoma. One of the other carcinomas contained foci of squamous metaplasia.

The bladder tumors were all epidermoid carcinomas and were small. An example is shown by Fig. 3.

Pulmonary adenomas were found in 7 rats; they were usually multiple and small (see Fig. 2). If several blocks of lung from each rat had been sectioned routinely, the incidence of adenomas would doubtless have been found to be considerably higher. One rat had an extensive epidermoid carcinoma of the lung. No other possible primary site for this tumor was found. Moreover, the other lung was free of metastases. Six rats were found to have metastatic pulmonary tumors; the primary sites of these tumors were: acoustic duct; ileum; liver (2 cases); and mammary (2 cases, 1 of which was the epidermoid carcinoma).

Portions of exorbital lachrymal glands or parotid glands were included in sections of several acoustic duct tumors. In no case did tumors develop in these glands, but in five instances the structure of the glands was strikingly altered. Nuclei were enlarged to a variable degree, and were often greatly enlarged. The amount of chromatia was considerably increased; the nuclei were stained deeply and often had large nucleoli. Some cells contained more than one nucleus. Occasionally, nuclei were crescentic and partially enveloped large vacuoles. In view of the observations of Heiman and Meisel (6), it is unfortunate that the salivary glands were not all sectioned routinely, and it is recommended that such measures be taken in future investigations. The salivary glands of our rats were not appreciably enlarged, and we had no reason to suspect neoplasia in them. Since the conditions of our experiments were entirely different, the findings should not be construed as contradictory.

Changes in the liver warrant a more detailed discussion than has been given of the other tissues. Enlargement of the liver was observed in about a third of the rats, and was due to hypertrophy of liver cells. Hypertrophy of the liver was also observed by Wilson and his associates (9). Well developed nodular cirrhosis was seen in only 5 rats, but in 20 other animals the presence of slight cirrhosis was revealed by microscopic examination, and in the livers of an additional 35 rats there were foci in which thin fibrous tissue trabeculae radiated outward and partly surrounded a few liver lobules. The last mentioned condition is evidently an incipient cirrhosis, for extension of the process would ultimately lead to the development of true cirrhosis. In addition to increase in fibrous tissue, there was usually some increase in small bile ducts. In some places there was considerable proliferation of bile ducts with formation of clusters of ducts. Many of these bile ducts were dilated and formed cysts of various sizes. The lining cells of some cysts were greatly

DESCRIPTION OF FIGURES 6 TO 9

Fig. 6.—Liver—multiple cysts. Mag. × 140.

Fig. 7.—Liver, malignant hepatoma with broad cords of cells. Mag. × 130.

Fig. 8.—Liver, malignant tumor containing glandular and trabecular elements. Mag. × 130.

Fig. 9.—Liver, malignant hepatoma with a region of acinus formation. Mag. × 140.
In places, such cysts were separated by small numbers of normal liver cells, as illustrated by Fig. 6. In many rats there was no increase in collagenous tissue in the liver. Except for hypertrophy, the changes just described are similar to those seen in the livers of rats treated with p-dimethylaminoazobenzene. Other differences observed were: (1) that the cysts in the livers of the rats treated with the fluorene compounds contained bile, a fact rendered obvious by fixation of the tissues, whereas the cysts in the livers of rats treated with p-dimethylaminoazobenzene did not; and (2) in livers of p-dimethylaminobenzene-treated rats a lesion which Opie (8) has named cholangiofibrosis was common, but was not encountered in rats treated with the fluorene derivatives.

In 12 rats only one liver tumor was present, and in seven instances it was a benign hepatoma. All other rats' livers contained 2 or more tumors. Some were benign hepatomas, and 3 were adenocarcinomas, but the majority were malignant hepatomas (as judged by invasion of veins or of liver tissue). In many, the cells were arranged in cords, 1 to 2 cells wide, but in some instances the cords were much broader, and, not infrequently, foci were seen in which some tumor cells were arranged about lumina. The proportion of tumors composed of cells with acidophilic cytoplasm was a little greater than in our p-dimethylaminoazobenzene tumors, but in general the tumors were quite similar. Examples are given in Figs. 7 to 9.

DISCUSSION

Since the site of tumor development in our rats differed so strikingly from that of Wilson and his co-workers (9), who used rats of the Slonaker strain, and agreed so well with that of Bielschowsky (2), who used rats of the same ancestry as ours, it appears that the incidence of tumors to be expected in the various tissues is determined by the strain of the rat employed.

The failure of the dietary modifications in these experiments to influence development of liver tumors indicates that the mechanism of tumor formation by the fluorene derivatives differs from that concerned with p-dimethylaminoazobenzene carcinogenesis. The ability of the former compounds to produce tumors in such a multiplicity of sites, as contrasted with the ability of the latter compound to produce liver tumors only, also suggests that the mechanism of tumor formation by the two classes of compounds is different.

There was no significant difference in tumor incidence or types of tumors developing in rats treated with 2-aminofluorene and 2-acetylaminofluorene.

SUMMARY

1. Addition of liver extract to the diet had no appreciable effect upon the production of liver tumors in rats by 2-acetylaminofluorene, and variation in the protein content of the diet had no evident effect upon the production of liver tumors by 2-aminofluorene and 2-acetylaminofluorene.

2. The site at which tumors may be induced by these two compounds is probably determined by the genetic constitution of the rat employed.

REFERENCES


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