Enhancement of Urinary Bladder Tumorigenesis in Hamsters by Coadministration of 2-Acetylaminofluorene and Indole

Ryoichi Oyasu, Takuya Kitajima, Martin L. Hopp, and Hirotoshi Sumie

Department of Pathology, Northwestern University Medical School, Chicago, Illinois 60611

SUMMARY

Hamsters treated with 2-acetylaminofluorene (AAF) were studied to test the effects of dietary indole and excess DL-tryptophan and the initial age of the animals on the development of bladder tumors. Neonatal males and females were given i.p. injections of AAF, 5 mg/100 g body weight, 3 times weekly until weaning. They were then fed a synthetic diet containing AAF with or without 1.6% indole or 2.0% DL-tryptophan. Enhancement of tumorigenesis in the urinary bladder was evident only when indole was added to the diet containing a low dose of AAF (0.03%). Twenty-four of 27 hamsters fed the combination diet for 10 months developed tumors (89%); 20 of them were invasive. On the other hand, 13 of 26 on the diet containing AAF alone developed bladder tumors (50%); 8 of them were invasive. The difference in incidence was significant \( p < 0.01 \). Development of bladder tumors appeared slightly delayed when older hamsters (initially 4 weeks old) were used, but after 11 months all animals developed bladder tumors. As was observed in rats, added indole or tryptophan protected the liver from AAF injury and greatly reduced the development of cholangiocarcinomas.

INTRODUCTION

The Syrian golden hamster will develop a very high incidence of bladder tumors after neonatal treatment with i.p. injections of AAF followed by a diet which contains AAF and 1.6% indole (11). Also, we have shown in rats (9) that the age of the animals at the beginning of AAF treatment affects the incidence of bladder tumors. Thus, 90% of the rats showed bladder tumors when the AAF treatment was begun at the age of 1 day, while only 10% developed tumors when such treatment was not begun until the rats were 2 weeks old. In the present study, we attempted to determine the role of dietary indole and excess DL-tryptophan on AAF bladder carcinogenesis in the hamsters, as well as the influence of the initial age of hamsters on the incidence of bladder tumors.

MATERIALS AND METHODS

Animals

Young Syrian golden hamsters from the stock generously provided by Dr. P. Shubik of the Eppley Institute for Research in Cancer, Omaha, Neb., were randomly bred in our laboratory.

Chemicals

AAF was purchased from Aldrich Chemical Co., Milwaukee, Wis., indole from Eastman Organic Chemicals, Rochester, N. Y., and DL-tryptophan from Nutritional Biochemicals, Cleveland, Ohio.

Diet

The dietary items were purchased from Nutritional Biochemicals, except for dextrin and casein, which were obtained, respectively, from the A. E. Staley Co., Decatur, Ill., and the National Casein Co., Chicago, Ill. The synthetic diets containing AAF and indole (or DL-tryptophan) were generally patterned after Diet 32 of Dunning and Curtis (5) but with a higher pyridoxine concentration (10 \( \mu g/g \) of diet). For the control diets the amount of dextrin was increased to replace the chemicals used for the experimental diets.

Experimental Plan

The following 3 major groups were established.

Testing the Role of Indole and Excess DL-Tryptophan on AAF Carcinogenesis. Within 24 hr after birth, the newborn males and females were given i.p. injections of AAF (5 mg/100 g body weight) suspended in 10% gum acacia 3 times weekly as described previously until the hamsters were weaned at 3 weeks of age (11). The control animals received vehicle only. Following weaning the animals were divided into the following groups: Group 1, initially, 18 males and 15 females that were fed a synthetic diet containing 0.03% AAF and 1.6% indole; Group 2, initially, 15 males and 15 females that were fed a synthetic diet containing 0.03% AAF only; Group 3, initially, 55 males and 45 females that were fed a synthetic diet containing 0.06% AAF and 1.6% indole; Group 4, initially, 39 males and 16 females that were fed a synthetic diet containing 0.03% AAF only; Group 3, initially, 55 males and 45 females that were fed a synthetic diet containing 0.06% AAF and 1.6% indole. Group 4, initially, 39 males and 16 females that were fed a synthetic diet containing 0.06% AAF and 2.0%...
DL-tryptophan. Casein hydrolysate was used in this diet instead of the regular casein used in our other diets. The total DL-tryptophan content was 2.2% and therefore is approximately 10 times that of other diets.

Testing the Effect of Delayed Onset of AAF Treatment on AAF Carcinogenesis. Group 6 was composed initially of 29 males and 14 females, which were given no i.p. injections, but, when 4 weeks old, were fed a synthetic diet containing 0.06% AAF and 1.6% indole.

Control Groups. The following groups of animals received i.p. injections of 10% gum acacia 3 times a week for 3 weeks until weaning. The diet thereafter contained no AAF. Then they were divided into the following groups: Group 7, initially, 23 males and 30 females that were fed a synthetic diet containing 1.6% indole; Group 8, initially, 18 males and 23 females that were fed a synthetic diet containing 2.0% DL-tryptophan; Group 9, initially, 21 males and 25 females that were fed a synthetic diet without AAF, indole, or excess DL-tryptophan.

Three animals were housed together in a plastic cage with free access to diet and water. The animals were weighed once a month; whenever any appeared moribund they were killed. Most of the animals of the experimental groups were killed at the end of 10 months or 10 months and 2 weeks of treatment, but some from Group 6 were allowed to live for 12 months of treatment. Most of the animals of the control groups were killed at the end of 12 months of dietary treatment. However, 18 animals from Groups 9 (no additives) and 7 (indole) were killed after 15 to 16.5 months of treatment. The animals killed and those dying spontaneously were autopsied for gross changes, and sections were taken routinely only from the liver and bladder.

Examination of Bladders

In the present study most bladders were not distended with fixative. Instead, they were opened along the midline and stretched on a piece of cardboard with 4 pins at the corners. Following fixation, the bladder was cut into 3 pieces, and all pieces were submitted for microscopic examination. All bladders were carefully examined for parasites under a dissecting microscope before being submitted to microscopic examination. Three microscopic slides were routinely taken from different levels of a block. In this study an attempt was made to distinguish carcinoma in situ from dysplasia, although admittedly the distinction between these 2 conditions is not clear and decision as to diagnosis may depend upon subjective interpretation. The results were therefore interpreted with or without inclusion of carcinomas in situ. Invasive carcinomas were divided into 3 stages according to the classification of Bonser and Jull (1). Only those animals that survived more than 8 months of treatment were included for final tabulation of the results.

RESULTS

General

Approximately 60% of the hamsters neonatally treated with AAF and subsequently fed the diets containing 0.06% AAF survived 8 months of treatment, while those fed diets containing 0.03% AAF or those whose dietary treatment with 0.06% AAF was delayed until the age of 4 weeks showed a better survival rate (approximately 80%). In all groups of animals, mortality was high in the 1st month following weaning. It was highest in Group 3 (0.06% AAF and indole), and 80% of deaths occurred within the 1st month of dietary treatment. The high mortality was ascribed in part to toxicity of AAF but largely to starvation; apparently, the indole contained in this diet was not palatable to animals. Deaths thereafter in all experimental groups were in part attributable to chronic diarrheal disease of unknown cause (so-called wet tail syndrome). The survival rates for the noncarcinogenic dietary groups ranged from 37 to 70%. The highest mortality was observed in Group 9 (no additives) and this appeared to be due to higher susceptibility to chronic diarrheal disease when the diet contained neither AAF, nor indole, nor excess tryptophan. Several animals from both the experimental and control groups were killed before the 9th month for the electron microscopic study of bladder.

The animals of all groups gained weight progressively for the first 7 to 8 months, but thereafter weight remained constant (Chart 1).

Pathology of the Experimental Animals

Since one of the primary objectives of the present studies was to critically evaluate the role of indole on the bladder neoplasia and it is known that the duration of exposure to carcinogen is an important factor affecting the incidence of bladder tumors, the number of animals in the various groups was kept equal if at all possible. Therefore, whenever animals fed AAF alone were killed, animals from the other group (AAF and indole) were randomly selected and killed for comparison.

Pathology of Bladders

General. Hematuria of AAF-fed animals began to appear at the end of 9 months and was more common among animals for which diets were supplemented with indole. In some, massive hematuria was noted terminally. No parasites were found in the bladder mucosa of any animals when examined under a dissecting microscope. There were no exophytic lesions and the mucosa was smooth and focally hyperemic. In those animals killed after 10 months and 2 weeks, punctate hemorrhage was frequently observed in the mucosa.

Microscopically, all animals examined after 8 months of AAF treatment revealed varying degrees of diffuse hyperplasia, as was previously observed (11). The lining cells were orderly arranged in this zone. Invasive carcinomas were divided into 3 stages according to the classification of Bonser and Jull (1). Only those animals that survived more than 8 months of treatment were included for final tabulation of the results.

*Casein hydrolysate, enzymatic, from Nutritional Biochemicals. This casein, according to their analysis, contains 0.9% DL-tryptophan.
epithelium as well as in the upper lamina propria became engorged and appeared increased in number. Within 1 month, dysplastic nodules of epithelial cells formed along the basal layer and extended into the lamina propria with smooth expansile borders. The component cells were highly atypical with squamous metaplasia, and mitotic figures were frequently observed. When atypia was marked, these cell nests were interpreted as carcinomas, which were preinvasive (Fig. 1). Proliferation of the intraepithelial capillaries continued and after 10 months led to erosion of the covering epithelium. The erosion of the epithelium was more common in the nonneoplastic than the neoplastic portions and resulted in rupture of these capillaries and massive hematuria. In animals with a 2- to 3-month history of intermittent hematuria, there were healed ulcers as evidenced by granulation tissue with chronic inflammatory cells in the lamina propria covered by an attenuated regenerated transitional epithelium. Animals with terminal massive hematuria always showed acute ulcers. In 3 instances, microcalculi were seen at the site of ulcer. Aggregates of chronic inflammatory cells, mostly lymphocytes but occasionally plasma cells as well, were frequently present in animals fed AAF with or without added indole or DL-tryptophan.

Meanwhile, many of the atypical cell nests described above extended further downwards, forming invasive nests. At the same time, branching nests of deeply basophilic cells developed at multiple foci and invaded the lamina propria (Fig. 2). As these nests grew, the cells formed inside the basal-type cells showed squamous differentiation, although intercellular bridges or keratinization was not observed. A great majority of bladder tumors were Stage 1 carcinomas. Stage 3 carcinomas (invasion of the adventitia) were observed only in 2 animals of Group 6 (0.06% AAF and indole, initially 4 weeks old) animals when killed at 12 months. No metastasis of tumor to the regional nodes or the distant organs was demonstrated.

**Bladder Tumors. Effect of Indole.** Effect of indole on the bladder tumorigenesis was clearly demonstrated when it was added to the diet containing a low level of AAF. Thus the animals of Group 1 (0.03% AAF and indole) showed a higher incidence of bladder tumors (89%) than those of Group 2 (0.03% AAF alone) (50%) (Table 1). The difference was statistically significant ($p < 0.01$). If invasive bladder tumors only were considered for tabulation, the difference in incidence between the 2 groups was again statistically significant ($p < 0.01$). On the other hand, among animals fed diets containing 0.06% AAF with (Group 3) or without (Group 4) indole there was no significant difference in incidence of tumors between these 2 groups (83% for the indole group and 71% for the AAF only group). At either carcinogenic level there was no significant difference in incidence of tumors between the males and females.
Table 1

Incidence of bladder tumors in hamsters

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Indole or tryptophan</th>
<th>Age at onset</th>
<th>No. of animals</th>
<th>Bladder tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Initial</td>
<td>Stage 0</td>
</tr>
<tr>
<td>Group 1</td>
<td>0.03 AAF</td>
<td>30</td>
<td>33</td>
<td>4</td>
</tr>
<tr>
<td>Group 2</td>
<td>0.03 AAF</td>
<td>30</td>
<td>30</td>
<td>5</td>
</tr>
<tr>
<td>Group 3</td>
<td>0.06 AAF + indole</td>
<td>40</td>
<td>100</td>
<td>6</td>
</tr>
<tr>
<td>Group 4</td>
<td>0.03 AAF + indole</td>
<td>30</td>
<td>40</td>
<td>5</td>
</tr>
<tr>
<td>Group 5</td>
<td>0.06 AAF + tryptophan</td>
<td>50</td>
<td>30</td>
<td>5</td>
</tr>
<tr>
<td>Group 6</td>
<td>0.06 AAF + indole</td>
<td>40</td>
<td>50</td>
<td>3</td>
</tr>
<tr>
<td>Group 7</td>
<td>0.06 AAF</td>
<td>40</td>
<td>30</td>
<td>0</td>
</tr>
<tr>
<td>Group 8</td>
<td>0.06 AAF</td>
<td>40</td>
<td>30</td>
<td>0</td>
</tr>
<tr>
<td>Group 9</td>
<td>0.06 AAF</td>
<td>40</td>
<td>24</td>
<td>0</td>
</tr>
</tbody>
</table>

a NB, newborn; 4W, 4 weeks.

Effect of Excess DL-Tryptophan. The incidence of bladder tumors in animals fed a diet with excess DL-tryptophan (Group 5) was 68%, and therefore excess DL-tryptophan did not enhance the incidence of bladder tumors.

Effect of Initial Age of Animals. The incidence of bladder tumors in animals initially 4 weeks old (Group 6) was 79%. The difference in incidence between the neonatal and 4-week-old groups was not statistically significant. Although not shown in Table 1, the tumor incidence rose to 100% when the animals survived more than 11 months.

Pathology of the Liver

Cholangiofibrosis developed in all animals which were fed the 0.06% AAF diets (Table 2). Depending on the severity of fibrosis and proliferation of bile duct cells, it was arbitrarily divided into 2 grades. Cholangiocarcinomas also developed in varying frequency. Many of the cholangiocarcinomas developed in the livers with Grade 2 cholangiofibrosis. The presence of indole in the diet significantly affected the development of the neoplastic and nonneoplastic liver diseases. The incidence of Grade 2 cholangiofibrosis and cholangiocarcinoma was much lower in Group 3 (0.06% AAF and indole) than in Group 4 (0.06% AAF only), and the differences in incidence were, respectively, statistically significant (p < 0.001). The effect of added indole was more striking at the 0.03% AAF level; cholangiofibrosis was found in all 26 animals of Group 2 (AAF), while only 7 of 27 animals of Group 1 (AAF and indole) showed cholangiofibrosis and in the remaining animals the changes were insignificant. None of the animals of Group 1 showed cholangiocarcinoma, but 4 of Group 2 had such malignancy.

The protective effect of excess tryptophan on the liver disease was also demonstrated. When compared with the results from Group 4 (AAF only), the differences were again statistically significant (p < 0.001 for cholangiofibrosis and p < 0.01 for cholangiocarcinoma).

Benign and Malignant Lesions at Other Sites

As in the previous study (11), peliosis was observed in a low yield. It was mostly in the spleen but was also found occasionally in the liver. It was found in 9 animals of Group 3 (0.06% AAF and indole), 4 of Group 4 (0.06% AAF), 10 of Group 6 (initially 4 weeks old, 0.06% AAF and indole), and 1 of Group 2 (0.03% AAF). In 1 animal from Group 6, blood loss due to rupture of a splenic peliosis was the cause of death.

Also found were papillary hyperplasias, papillary adenomas, and papillary adenocarcinomas of the gallbladder. Most of them were benign lesions and were distributed evenly among AAF-treated animals. Other tumors observed were 2 invasive transitional cell carcinomas of the ureter, one each in Group 6 (AAF and indole, 4 weeks old initially) and Group 2 (0.03% AAF), an adenocarcinoma of the common bile duct, and an adenocarcinoma of the pancreas of Group 4 (0.06% AAF).

Seven hamsters treated with AAF (both 0.03 and 0.06% levels) with or without indole or tryptophan developed arteritis with fibrinoid necrosis and inflammatory exudate in the adventitia of the bladder. None of the control animals developed arteritis.

Pathology of the Control Animals

No tumors were found in the control animals (Groups 7, 8, and 9). Scattered foci of mild chronic inflammation of the...
Bladder mucosa were found in 12 out of 30 Group 7 (indole), 8 out of 35 Group 8 (tryptophan), and 2 out of 17 Group 9 (no additives) animals.

**DISCUSSION**

Bladder cancer study was greatly stimulated when Dunning and Curtis (5) and Dunning et al. (6) first demonstrated that excessive DL-tryptophan or indole added to a synthetic diet containing 0.06% AAF increased the incidence of bladder tumors in female Fischer rats. Subsequent studies were divided into p.o. testing of indole, tryptophan, or its intermediary products with the use of rats, mice, and dogs, and direct testing by inserting into bladder lumens the pellets containing these test compounds. The results of subsequent studies p.o. by other investigators are conflicting. One of our previous studies (10) showed a higher incidence of bladder tumors in groups of rats with added indole than in control rats fed a diet supplemented with AAF alone; however, the survival time of the group receiving AAF alone was too short to allow the role of indole on bladder tumorigenesis to be assessed. The data were adequate to show that indole definitely decreased AAF hepatotoxicity and hepatocarcinogenicity, and we concluded that prolonged survival time was a probable decisive factor causing the increase in the bladder neoplasia by AAF. Other workers studying this problem, such as Boyland et al. (2) in experiments with female Wistar rats, confirmed the enhancing role of tryptophan on the AAF bladder tumorigenesis, but their studies also suffered from early deaths of the AAF control animals. Morris et al. (8) studied the effects of tryptophan given by different feeding schedules. They fed a diet containing 0.06% AAF and 1.4% DL-tryptophan to Fischer rats alternately at weekly intervals with a similar diet devoid of AAF. The effects of added tryptophan could not be demonstrated in female rats because all animals, whether tryptophan was added or not, developed bladder tumors, while the results obtained in male rats appeared to support Dunning’s conclusion. On the other hand, Chapman (4), while testing the effect of parasite, *Trichosomoides crassicauda*, on the AAF bladder tumorigenesis, saw only 1 bladder tumor in the 17 male and 24 female Fischer rats fed a diet containing 0.05% AAF and 1.0% DL-tryptophan. The variable incidence of bladder tumors described above might be due to differences in pyridoxine level in the diet which is known to alter tryptophan metabolism quantitatively. In all these studies except the one by Chapman, whether the results were positive or negative, the interpretation of the results was made difficult because of the small number of animals tested. Since there are many reports implicating tryptophan intermediary metabolites as bladder carcinogens when tested by pellet implantation to the bladder lumens of mice (3), it was of utmost importance to confirm Dunning’s results. To test this, rats were obviously not an ideal species because of a high incidence of and, therefore, high mortality from hepatocellular carcinomas.

One of our previous works (11) has clearly shown that Syrian golden hamsters were suitable for this study because of the high incidence of malignant tumors almost restricted to the bladder.

Our present investigation was performed with 2 levels of AAF, 0.03% and 0.06%. With regard to bladder tumors, our results showed that added dietary indole had no effect when AAF was fed at 0.06%. When the dose of AAF was reduced to 0.03%, however, there was a definite difference in the tumor incidence. Thus in Group 2 (AAF alone), only 13 out of 26 hamsters (50%) developed bladder tumors, while, in Group 1 (AAF and indole), 24 out of 27 hamsters (89%) developed bladder tumors. The difference was statistically significant ($p < 0.01$).

The present experimental results have not only clearly confirmed the enhancing effect of indole on the AAF bladder tumorigenesis first demonstrated by Dunning and Curtis (5), but also the fact that the enhancing effect of indole became evident only when a lower level of AAF in the diet was used.

With regard to hepatic effects of AAF, the protective effect of indole or excess DL-tryptophan on the AAF hepatotoxicity and carcinogenicity was clearly demonstrated at both the high and low levels of carcinogen. The effect of indole or excess DL-tryptophan on the liver observed in the present study was similar to what we observed in Wistar rats (10), and we conclude that indole definitely reduced the oncogenicity of AAF to the liver. Dunning and Curtis (5) and Dunning et al. (6) did not detect this protective action in rats, and we have no explanation for this discrepancy other than to speculate that differences in sex, strain, and species may be responsible. The mechanism of the protective action of indole to the liver is not clear. It may be related to the decreased binding of $N$-hydroxy-AAF to the RNA and DNA of the liver cells (7). Those animals fed the diets fortified with indole weighed less than those fed the diets without it. Although dietary consumption was not measured, this may indicate that the effect of indole may be due to decrease of the intake of AAF and of calories during the early period of the experiment, thereby altering the AAF metabolism in the liver, rather than to a direct effect of indole on either the metabolism or the bladder epithelium. On the other hand, the role of indole or excess tryptophan may be that of a promoter. If that is the case, the effect of indole may become more overt when it is added to the diets containing lower levels of carcinogen. This hypothesis is being tested in our laboratory.

**REFERENCES**


R. Oyasu, T. Kitajima, M. L. Hopp, and H. Sumie


Fig. 1. Marked downward growth of highly atypical bladder epithelium with squamous metaplasia. These nests show a smooth outer border and expansile mode of growth and are considered to represent preinvasive carcinoma. Animal from Group 3 (0.06% AAF and indole), killed after 9 months and 2 weeks of carcinogenic treatment. H & E, × 105.

Fig. 2. Multiple nests of invasive carcinoma. The cells in the outermost layer are small and basophilic. The mucosal surface is eroded. A small number of chronic inflammatory cells are present. Animal from Group 3 (0.06% AAF and indole), killed after 10 months of carcinogenic treatment. H & E, × 105.
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