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4-ETHYLSULPHONYLNAPHTHALENE-1-SULPHONAMIDE: A NEW CHEMICAL FOR THE STUDY OF BLADDER CANCER IN THE MOUSE

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Abstract—4-Ethylsulphonylnaphthalene-1-sulphonamide has been shown to be carcinogenic to the bladder epithelium of the C57 × IF mouse. Female mice (44 per cent carcinomas) were more susceptible than males (16 per cent), while male (21 per cent) and female (28 per cent) castrates occupied an intermediate position. The hyperplasia induced by the chemical after 2 or 6 weeks administration is correlated with the ultimate incidence of tumours in the mice of the differing hormonal states. It is shown that the number of mitoses in the bladder epithelium of the A × IF mouse is raised about ten times at 6 and 12 hr and more than 200 times at 24 and 30 hr after the administration of a single dose of the chemical. These facts, with others which have been established previously, are held to support the idea that in the mouse the induction of bladder cancer depends on the presence of a carcinogenic factor in combination with the stimulation of the epithelium to mitosis.

THE STUDY of bladder cancer has been limited by the relatively small number of chemical agents which, after systemic administration, lead to the pathological condition. In the mouse, Armstrong and Bonser[1] showed that 2-acetylaminofluorene was effective in inducing cancer of the urinary bladder in each of the five inbred strains which they investigated. 4-Aminodiphenyl induced only a small number of tumours in Ab × IF[2] and C57 × IF[3] F_1 hybrid mice surviving for more than 80 weeks. 2-Aminodiphenylene oxide[3] was effective in male C57 × IF mice but the females died early from liver tumours. The discovery that the oral administration to mice of 4-ethylsulphonylnaphthalene-1-sulphonamide (HPA)[4, 5] led to carcinomas of the bladder epithelium was of more than usual interest and this was heightened by the fact that the three aromatic amines, already mentioned, gave tumours in other tissues, especially the liver, whereas HPA was confined in its action to the bladder epithelium.

HPA was originally synthesized by Brimelow and Vasey[6] as an anticonvulsant and diuretic, but was prevented from undergoing clinical trial because it induced epithelial hyperplasia of the bladder after oral administration to the rat.[7] Sen Gupta[8] showed that certain types of mouse were similarly affected but the dog and guinea pig were resistant. More recent work has shown that the rabbit was apparently not susceptible[3] and that the hamster developed epithelial hyperplasia less readily than the mouse.[2] Long term feeding of HPA to Ab × IF F_1 hybrid mice led to papillomas and carcinomas of the bladder epithelium which were significantly more frequent in female than in male mice.[4, 5]

In this report it is intended to present new evidence on the carcinogenicity of HPA and on the correlation between early hyperplasia and malignancy. The earliest changes induced by the chemical will be described. In conclusion an attempt will be made to indicate the relevance of these and other findings to the mechanism of bladder carcinogenesis.

MATERIALS AND METHODS

Chemicals

4-Ethylsulphonylnaphthalene-l-sulphonamide (HPA) was prepared in the laboratory.[6]

Animals

Mice of inbred strains and their F_1 hybrids were bred in the laboratory. Control mice were maintained on pelleted Oxo Diet 41B and water *ad libitum*. HPA in acetone was mixed thoroughly with powdered Oxo Diet 41B containing enough water to make a stiff dough. The dough was flattened into a thin biscuit and dried in an oven at 56°. This biscuit and water *ad libitum* were administered to the experimental mice. Castration was carried out, if required, at least 15 days before the start of chemical treatment.

Animals which were sick or had survived to the end of the experiment were killed with ether, the bladder distended with Bouin's fluid and prepared in the usual manner for histology.[8] Animals which died were discarded, as the bladder epithelium was autolysed. Carcinomas were graded by previously described criteria.[9]

Mitotic counts

In these experiments, 500 μg HPA in arachis oil (0·2 ml) was administered at 10 a.m. by stomach tube to young adult A × IF F_1 hybrid mice, followed in half of the animals by the subcutaneous injection of colchicine (0·03 mg) in saline (0·2 ml) 5 hr before death. Two sections (4–5 mμ thick) separated by 250 mμ were taken from each half of the bisected bladder and were stained with haematoxylin and eosin for mitotic counting. Each section contained about 1000 epithelial cells and approximately 1000–2500 cells/mouse were examined. A section of caecum was prepared in each case as a control.

RESULTS

Hyperplasia

It was desired to determine whether 4-ethylsulphonylnaphthalene-1-sulphonamide induced a greater degree of hyperplasia in female mice, which are more susceptible to the induction of bladder tumours by this chemical, than in male mice. Groups of eight male or female young adult C57 × IF mice were maintained for 6 weeks on a diet containing either 0·01, 0·005, 0·0025 or 0·0013% HPA. The hyperplastic index for each group was determined by scoring five points for a bladder showing large areas or foci of epithelium which was more than five cell layers thick, three points for a mildly hyperplastic bladder with three to five cell layers and one point for a doubtfully hyperplastic epithelium. The normal distended mouse bladder epithelium appears under the light microscope to be two cell layers in thickness. The total of

points (N) for the whole group was calculated and the hyperplastic index evaluated using the expression $\frac{20N}{n}$, where n is the number of animals in the group. From Fig. 1 it is apparent that HPA is more effective in inducing hyperplasia at concentrations of 0·005, 0·0025 and 0·0013% in female than in male C57 × IF mice, but at 0·01% it is equally effective in either sex.

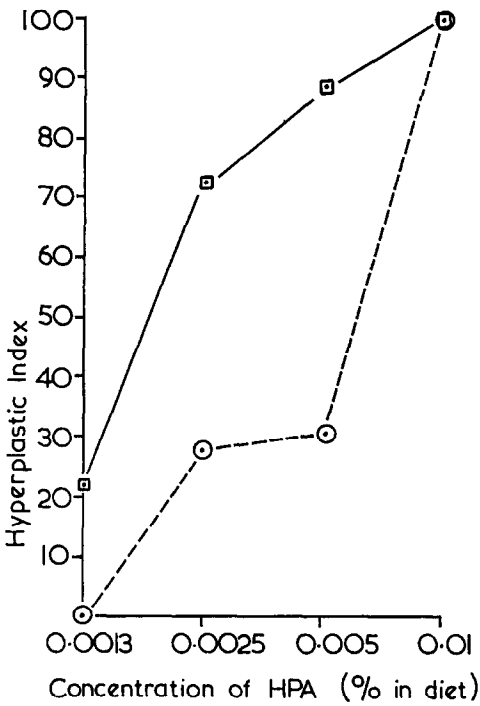

FIG. 1. Effect of the concentration of HPA in the diet on the incidence of hyperplasia of the bladder epithelium in female (⊡———⊡) and male (⊙- - - -⊙) C57 × IF mice, 6 weeks after the start of treatment.

In a second experiment, groups of six IF mice were maintained in the same way for 2 weeks on diets containing 0·02, 0·01, 0·005, 0·0025 and 0·0013% HPA with the results shown in Fig. 2. Once again female mice were more susceptible to the induction of hyperplasia than were male mice. Castrated female mice tended to resemble intact females and castrated males were similar to intact males. The hyperplasia was less severe and more variable than in the first experiment either because of its shorter duration or because a different kind of mouse was used.

Carcinogensis experiments

Female Ab × IF mice are more susceptible than males to bladder carcinogenesis by HPA.[4, 5] We therefore determined the influence of castration on the tumour incidence (Table 1). In the course of setting up the experiment, the Ab substrain failed to breed and was lost. In consequence the number of animals used was small. Four of six female castrates and nine of ten male castrates surviving between 30 and

65 weeks on a diet containing 0·01% HPA developed carcinomas of the bladder epithelium. The high incidence of tumours in castrate males was unexpected but the number of animals is too small to allow any significant comparisons between the groups.

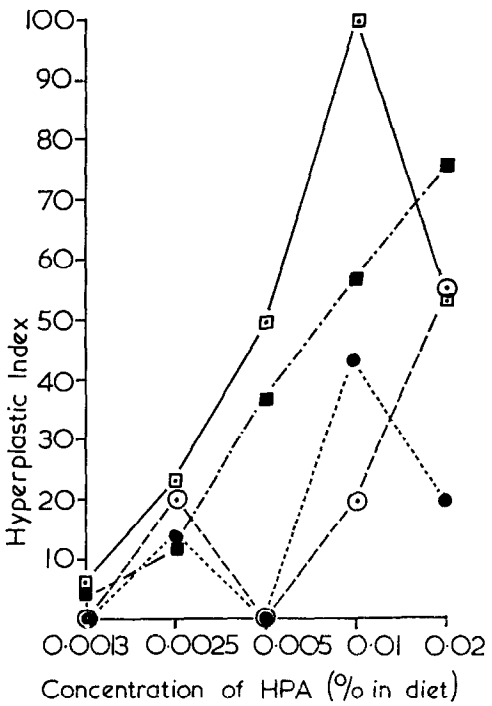

FIG. 2. Effect of the concentration of HPA in the diet on the incidence of hyperplasia of the bladder epithelium in female (□———□) female castrate (■ · - - - · ■) male castrate (● · · · · ●) and male (⊙- - - -⊙) IF mice, 2 weeks after the start of treatment.

TABLE 1. EFFECT OF CASTRATION ON INDUCTION OF TUMOURS OF THE BLADDER EPITHELIUM IN Ab × IF MICE BY HPA (0·01%) IN THE DIET

No. of mice surviving to 30 weeks	Sex	Squamous metaplasia	Papilloma*	Carcinoma*		
				I	II	%
34†	M	4	2	3	2	15
10	CM	3	0	5	4	90
39†	F	12	3	11	7‡	46
6	SF	3	0	2	2‡	67

* Most advanced lession only.
† From Clayson and Bonser.[5]
‡ Includes 1 grade III carcinoma.

We therefore repeated the experiment in C57 × IF F_1 hybrid mice which were known to be susceptible to the induction of early hyperplasia by HPA.[4] It was found (Table 2) that, on a diet containing 0·01% HPA, female mice (seven carcinomas in sixteen mice) were again more susceptible than male mice (two in thirteen). The

incidence with castrated male mice (four in nineteen) and castrated female mice (five in eighteen) was intermediate between that found in intact males and females.

HPA induces a phosphatic crystalluria in addition to hyperplasia in the urinary tract which leads to partial restriction of the urinary flow and, consequently, to

TABLE 2. EFFECT OF CASTRATION ON INDUCTION OF TUMOURS OF BLADDER EPITHELIUM IN C57 × IF MICE BY HPA (0·01%) IN DIET

No. of mice surviving to 30 weeks	Sex	Squamous metaplasia	Papilloma*	Carcinoma*		
				I	II	%
13	M	1	0	1	1	16
19	CM	0	1	4	0	21
16	F	2	0	6	1	44
18	SF	4	0	4	1	28

* Most advanced lesion only.

hydronephrosis and hydroureter. The early death of many animals from these causes means that the number of effective survivors between 30 and 65 weeks is small and none of the differences in tumour yield is significant at the 5 per cent level. The carcinomas were less malignant than those induced in Ab × IF mice. No tumours other than those of the bladder were found in these mice when the carcinogenesis experiments were terminated at 65 weeks.

Mitotic counts

Hyperplasia of the bladder epithelium is a relatively crude index of the earliest changes induced by a chemical. The histologically-apparent increase in the thickness of the epithelium may be caused either by an increase in the number of dividing cells, or by a diminution in the number of cells being discarded, or by a prolongation in the lifespan of the cells. A more precise approach to the earliest changes is the direct determination of cell turnover. Clayson and Pringle[10] found that there are very few mitoses in bladder epithelium from normal intact mice either with or without the prior administration of colchicine.

In this investigation, we administered a single dose of 500 μg HPA by stomach tube, in order to be able to define precisely the time of administration of the chemical. Groups of A × IF mice were killed at 6, 12, 24, 30 and 54 hr thereafter and their bladders examined for mitoses either with or without the administration of colchicine 5 hr before death. Six hours after the administration of the chemical (Table 3) the number of mitoses in the bladder epithelium had risen to approximately ten times the value found in intact mice. This was maintained at 12 hr when areas of necrosis were found in the epithelium. By 24 hr the number of mitoses rose to more than 200 times that found in the normal bladder. This high rate was maintained at 30 hr but declined slightly by 54 hr. Because of the variability between sections, which appeared to be influenced not only by variations between individual mice but also by the relation of the section to the areas of necrosis, a far larger series will have to be counted to determine whether there is a significant difference in the incidence of mitoses between male and female mice. Similarly it is premature to calculate the mitotic duration at different times after the administration of HPA.

TABLE 3. NUMBER OF MITOSES IN THE BLADDER EPITHELIUM OF A × IF MICE AT INTERVALS AFTER ADMINISTRATION OF 500 μg HPA IN ARACHIS OIL BY STOMACH TUBE

	Without colchicine					With colchicine				
Time after HPA (hr)	No. of mice	Sex	No. of cells (No. of sections)	No. of mitoses	No. of mitoses in 10^3 cells (range)	No. of mice	Sex	No. of cells (No of sections)	No. of mitoses	No. of mitoses in 10^3 cells (range)
0*	7 7	M F	71,459 (75)	4	0·056	7 7	M F	47,635 (45)	4	0·084
6	3 3	M F	10,807 (12)	4	0·37 (0–0·7)	3 3	M F	12,714 (18)	20	1·5 (1–2·8)
12	3 3	M F	6,890 (10)	3	0·4 (0–0·9)	3 3	M F	6,210 (10)	6	0·9 (0–1·6)
24	3 3	M F	7,546 (6)	128	16·9 (4·6–18)	3 3	M F	5,780 (6)	236	40 (34–60)
30	3	F	3,140 (6)	20	7 (4–13)	3	F	5,675 (6)	273	52·8 (28·4–68·4)
54	3	M	3,801 (5)	18	4·7 (1·4–7)	3	M	4,894 (5)	128	26 (7·7–38)

* From Clayson and Pringle.[10]

DISCUSSION

4-Ethylsulphonylnaphthalene-1-sulphonamide (HPA) is carcinogenic to the bladder epithelium of C57 × IF mice as well as of Ab × IF mice. In both cases the intact females were more susceptible than the intact males. In the C57 × IF mice the incidence of tumours in male and female castrates was intermediate between that found in intact female and intact male mice. In the Ab × IF mice, for reasons which have been explained, the number of survivors was too small to allow the effects of castration to be assessed. The fact that, at the lower concentrations of HPA in the diet, the early hyperplasia induced in female C57 × IF mice was greater than that in the male further supports the positive correlation between the chemical induction of early hyperplasia and ultimate malignancy of the bladder epithelium which was shown by Clayson *et al.*[2] The trend of the results with intact female and male IF mice lends further indirect support to the correlation; but the observation that castrated male and castrated female mice produced hyperplasia of a degree similar to that of the corresponding intact animals needs further study.

It is apparent from the results obtained in those experiments concerned with mitotic counts that the hyperplasia induced by the chemical is only a reflection of the early effects of HPA on the bladder epithelium. It is thought that the ten-fold increase in the number of mitoses at 6 and 12 hr after the administration of the HPA possibly represents a direct stimulatory effect of the chemical or its metabolites on the epithelium. The further increase in mitoses at 24, 30 and 54 hr is thought to be brought about by the necessity to repair the necrotic areas first observed at 12 hr. Walker[11] showed that repair, after mechanically wounding the bladder epithelium occurred between 1 and 4 days and involved a substantial increase in the number of mitoses. In the present experiment the chemical was administered by stomach tube whereas in most of the longer term experiments it was given in the diet. The effect of the method of administration on the mitotic activity in the bladder epithelium will have to be investigated.

It is now possible to define some of the factors which influence the course of carcinogenesis in the bladder. The young adult mouse bladder epithelium has very little mitotic activity.[10] Leblond *et al.*[12] demonstrated that in the rat, the activity was higher than we reported in the mouse, but Leblond (personal communication) subsequently found that "rats of the type used in these investigations frequently had parasites in the bladder and the presence of parasites raised the number of mitoses in the epithelium considerably. The uninfested rat bladder is comparable to the mouse and contains few mitotic figures." It seems likely that the bladder epithelium in these species needs to be stimulated to mitotic activity for carcinogenesis to ensue. This stimulation may be brought about by a chemical, as shown in this paper, or by the presence of a foreign body in the lumen of the bladder.[10]

It is probable that an increase in mitotic activity is not enough by itself and that a "carcinogenic" stimulus is also necessary. This could be provided by the systemic or local administration of a chemical carcinogen. Alternatively the carcinogenic stimulus might be provided by endogenous carcinogens, such as certain metabolites of tryptophan, in the urine.[13] Most of these supposed endogenous carcinogens have been identified by the method of bladder implantation[14] but Bryan and Springberg[15] showed that the subcutaneous administration of one tryptophan metabolite, the 8-methyl ether of xanthurenic acid (XAE), induced carcinomas of the mouse bladder

epithelium in mice implanted with unadulterated cholesterol pellets but not in unoperated mice. This suggests that the pellet and the XAE each contribute a part of the conditions necessary to induce cancer. It is a reasonable speculation that the influence of the pellet* is to induce mitosis and the XAE to provide the "carcinogenic" factor. Greater precision in the anlaysis of murine bladder cancer would result from the quantitation of the endogenous carcinogens in the urine of various strains of mice, and a comparison of the results with the incidence of tumours obtained in various conditions.

The effect of the sex of the mice on the number of tumours induced by HPA requires further investigation. It is not at present possible to decide whether the sex hormones act directly on the bladder epithelium or indirectly by altering the metabolism of the chemical. Work is also required to determine the factors contributing to the integrity and homeostatic control of the bladder epithelium.

* Cholesterol pellets increase the mitotic activity of the bladder epithelium[10].

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