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Rat Skin Carcinogenesis by Topical Applications of Some Azo Dyes[1]

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Summary

Groups of 6 male rats were painted twice weekly with 1 ml of a 0.2% solution of an azo dye in acetone. The dyes used were aminoazobenzene, 4-monomethylaminoazobenzene, their 3-methoxy analogs, and 4-dimethylaminoazobenzene. In each case, all rats developed multiple skin tumors of histologic types similar to those found in rats treated with other chemical classes of carcinogen. 3-Methoxy-4-monomethylaminoazobenzene was the most potent carcinogen, followed by 4-monomethylaminoazobenzene, 3-methoxyaminoazobenzene, and 4-dimethylaminoazobenzene, and finally aminoazobenzene.

3-Methoxy-4-dimethylaminoazobenzene, which is also a potent carcinogen for rat skin, was found to be totally ineffectual when painted on the skins of 280 mice.

Introduction

Fare and Orr (4) reported that 10 albino rats "painted" with an acetone solution of 3-methoxy-DAB[3] all developed multiple skin tumors with an average induction time of 46 weeks for the appearance of the 1st lesion. The tumors, all of which arose within the treated area of dorsal skin, were of the same histologic types that occur following treatment of rats with other skin carcinogens: squamous carcinoma, keratoacanthoma, and basal cell carcinoma, sometimes with sebaceous or trichoepitheliomatous differentiation.

No previous records could be found in the literature of rat skin tumors produced by azo dye painting, and so it was clearly of interest to repeat the experiments under identical conditions but using other related compounds. 3-Methoxy-AAB and 3-methoxy-MAB were tested to determine whether 1 or more *N*-methyl substituents are necessary for activity. Similarly, the 3 parent dyes—AAB, MAB, DAB—were also used to investigate whether a methoxy grouping on position 3 is necessary. At the same time, "control" animals were treated with acetone alone.

In addition, 3-methoxy-DAB was painted on mouse skin.The mouse is a species normally more susceptible to skin painting than the rat yet the latter species is the more susceptible as regards liver carcinogenesis when azo dyes are fed. A control group treated with acetone alone was not considered necessary, since this solvent is, in our experience, without effect on mouse skin insofar as tumor production is concerned.

Materials and Methods

CHEMICALS. AAB (British Drug Houses, Poole, England) and DAB (Hopkin and Williams Ltd., Chadwell Heath, England) were normal commercial products recrystallized before use. MAB (7), 3-methoxy-AAB (9), 3-methoxy-MAB (8), and 3-methoxy-DAB (9) were prepared and purified as described in the literature. All the dyes when purified for use in these experiments migrated as single spots when submitted to thin layer chromatography on Kieselgel in the solvents used by Topham and Westrop (10).

The solvent used was redistilled "Analar" grade acetone (Hopkin and Williams Ltd.). The solutions were made up fresh every 2 weeks.

ANIMALS. Six groups of 6 male rats, aged 3–4 months, of our albino, outbred stock were used, housed in galvanized wire mesh cages. These small groups were considered adequate, since 32 lesions were produced, on the average, in each animal treated with 3-methoxy-DAB in the previous experiment (4).

In the mouse-painting experiment, 140 albino, outbred mice of each sex were used, housed in plastic boxes holding 5 mice each.

Both rats and mice were given proprietary cube feed ("Thomson" diet) obtained from Messrs. A. R. Heygate and Sons, Bugbrooke Mill, Northampton, England, and tap water *ad libitum* throughout.

ASSAY PROCEDURE. All animals were painted twice weekly on the dorsal skin between the scapulae with a 0.2% solution of the appropriate dye in acetone (Table 1). The control rats received acetone alone. The volume applied was 0.2 ml on mice and 1.0 ml on rats. Hair was shaved every 2 months in the early stages but much more frequently when tumors began to appear. A record was kept of all tumors that arose, so that the progress of each lesion, e.g., regression, growth, coalescence with other tumors, could be evaluated.

Occasionally, rats were killed because of general ill-health or the development of "ear-duct" tumors, but the usual reason was the ulceration of skin lesions. The mice died of the various diseases attendant upon old age.

All tumors, together with surrounding skin, were removed and fixed in formaldehyde-saline for histology. Sections, cut at 5 μ, were stained with Ehrlich's hematoxylin and eosin and with Weigert's hematoxylin and Van Gieson.

A full postmortem examination was performed in every case; a histologic examination was performed on any tissue showing abnormality. Representative liver sections were taken in every case, even when the organ appeared normal.

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[3] The following abbreviations are used: AAB, aminoazobenzene; MAB, monomethylaminoazobenzene; DAB, dimethylaminoazobenzene; MeO-, methoxy group.

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TABLE 1

RESULTS OF PAINTING SKIN OF RATS AND MICE WITH AZO DYES

SPECIES	SEX	DYE	MEAN TIME 1ST LESION NOTED (wk.)	MEAN LENGTH OF TREATMENT (wk.)	TUMOR INCIDENCE		TOTAL YIELD OF SKIN TUMORS IN GROUP, POSTMORTEM[a]						
					Ear duct	Skin	Epidermoid cyst	Keratoacanthoma	Squamous carcinoma	Basal carcinoma	Anaplastic carcinoma	Squamous papilloma	Miscellaneous
Rat	M	None (Control)		131	0/6	0/6							
Rat	M	AAB[b]	97	123	0/6	6/6	5		4	8	2	1	3
Rat	M	MAB	44	58	0/6	6/6	16	3	7	18	9	2	7
Rat	M	DAB	73	90	0/6	6/6	2		3	11	4		3
Rat	M	3-MeO-AAB	76	93	1/6	6/6	3	1	3	5	5	2	5
Rat	M	3-MeO-MAB	28	41	2/6	6/6	27	2	12	28	13	2	6
Rat	M	3-MeO-DAB[c]	47	62	3/10	10/10	2	2	10	16	1	1	4
Mouse	F	3-MeO-DAB		30	0/140	0/140							
Mouse	M	3-MeO-DAB		62	0/140	0/140							

[a] Tumor types are described more fully in the text.

[b] Abbreviations: AAB, aminoazobenzene; MAB, monomethylaminoazobenzene; DAB, dimethylaminoazobenzene; MeO-, methoxy group.

[c] Data from Fare and Orr (4). The numbers of tumors produced are not comparable with those in the other treatment groups since in this particular case not all tumors were examined histologically.

All livers were assayed for free and protein-bound dye by the method of Fare (1).

Results

Mice

When the last survivor died after 26 months of treatment, no skin tumor had been found in any mouse nor were there any other abnormalities, postmortem, that could be ascribed to dye treatment. No dye could be detected in the livers. The male mice survived longer (Table 1) because the females frequently developed spontaneous breast cancer (a feature of our random-bred albino stock) and had to be killed.

Rats

All the rats grew satisfactorily and weight gains were identical with those found in untreated male rats of the same age. Tumor incidences are given in Table 1.

Tumors of the ear duct arose only in rats treated with any 1 of the 3-methoxy dyes. These tumors were invariably unilateral, in agreement with previous experience (2–4).

The skin tumors were of the histologic types associated with the action of other chemical classes of carcinogen on rat skin and with 3-Meo-DAB (4), and therefore they are not illustrated. Their macroscopic appearances were similar to those described previously for 3-Meo-DAB (4) and, as with this compound, about 50% of all lesions formed regressed during continued treatment. As the number of lesions increased, the amount of dye applied was lessened, so that only tumor-free areas of skin within the target area on the dorsum were treated.

An attempt has been made to allot each tumor found postmortem into 1 of 7 categories (Table 1).

A large majority of the cystic lesions were classified as simple epidermoid cysts, some of them opening to the surface and some of them multilocular. A few cysts showed sebaceous differentiation, and an occasional epidermoid cyst was found surrounded by trichoepithelioma.

Relatively few keratoacanthomas were found, an average of only 1 in each treatment group. They were slow-growing lesions which reached a large size by developing from a single focus. No keratoacanthoma was observed to develop by coalescence of adjacent multiple lesions, and their histologic appearance was exactly that described previously (5, 6).

Squamous carcinomas were relatively common, an average of 1 per rat when killed. They exhibited the well-known histologic pattern with invasion through the panniculus carnosus.

The largest category in Table 1 is the group of lesions classed as basal cell carcinomas. These included undifferentiated basal cell carcinomas and basal cell or basisquamous carcinomas showing differentiation to hair follicles or sebaceous cells including trichoepitheliomas. The undifferentiated tumors correspond with the "rodent type basal cell carcinoma" described by Howell (6), but where there was differentiation, a sharp distinction between "trichoepithelioma" and sebaceous tumor was often difficult, as with 3-Meo-DAB (4). This group of tumors often developed by multiple adjacent tumors coalescing into a single lesion, and the resulting large tumors showed a marked tendency to ulcerate.

A number of tumors were classified as anaplastic carcinomas when their origins were doubtful.

Seven squamous papillomas were found in the 30 rats. These arose in surface epithelium and did not penetrate the dermis.

The remaining lesions were all classed as "miscellaneous" and included 2 fibromas and 4 pleomorphic spindle cell sarcomas. The remainder were areas of noninvasive intraepidermal change.

No tumors were found postmortem at any other sites other than skin and ear duct, and no dye could be detected in livers. Hepatic tissue showed no histologic abnormalities.

Discussion

The different tumor types resulting from dye painting are relatively unimportant as far as this study is concerned. What is interesting is that the azo dyes unsubstituted on the 3-position

with a methoxy group are carcinogenic to rat skin. Hitherto, this class of compound has been regarded as carcinogenic exclusively to liver.

Even though only 6 rats were used in each group, it was obvious from a study of tumor induction times and the average number of lesions developing per rat during treatment that there was a difference in potency between the powerful 3-MeO-MAB and the slightly less active MAB [the tumor induction time of which was identical with that for 3-MeO-DAB reported previously (4)] and the other 3 dyes tested. The weakest of all appeared to be the parent compound, AAB.

Thus, it would seem that for both the series AAB, MAB, DAB, and 3-MeO-AAB, 3-MeO-MAB, 3-MeO-DAB, the dye with 1 methyl substituent on the 4-amino group is the most powerful, the dimethyl compound intermediate, and the unsubstituted dye least active. When each of the dyes AAB, MAB, and DAB is compared with its 3-methoxy analog, the introduction of the 3-methoxy group increases the potency.

Since all 6 dyes do, however, give a 100% tumor incidence, it should perhaps be stressed that each of the dyes purified for use migrates as a single spot when submitted to chromatography and that the redistilled acetone used as vehicle produced no damage when applied to rat skin for over 2 years in the control experiment. The differences in potency were therefore real and cannot be attributed to contamination of the dyes by activating or inactivating material. The metal rat cages, plastic mouse boxes, and all water bottle spouts were demonstrated not to have been contaminated before use by any of the carcinogens used in the laboratories.

Since no liver lesions or even the presence of dye in the livers could be demonstrated, it seems likely that the dyes affect the skin directly following topical application and not after oral assimilation of the dye, unless small amounts ingested by licking the skin and insufficient to give a positive result for liver dye content are sufficient to cause skin tumors.

Mice were found to be totally resistant to 3-MeO-DAB. This resistance is in line with an increased resistance by the mouse to liver carcinogenesis compared with that of the rat which is obtained following the feeding of azo dyes. It is an exception to the usual experience that topical skin carcinogens are more effective in the mouse than in the rat.

Skin from mice and rats treated with various azo dyes and 3-methoxy derivatives is being examined to try to determine whether the 2 species metabolize the various dyes in different ways, and to try to find the chemical nature of the active carcinogen(s).

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