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Induction of Pituitary Tumors in Germ-free Rats Exposed to *Aspergillus versicolor*

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ABSTRACT

We clarified the role of mold exposure in pituitary tumor induction using 53 germ-free Wistar rats exposed before birth and continually exposed thereafter, to *Aspergillus versicolor* alone. Rats were autopsied at 540 to 730 days. Untreated germ-free controls were autopsied on day 730. Thirty-three of the 53 monoassociated rats had pituitary tumors (62.3%), while only three of the 41 control germ-free rats had tumors (7.3%). It seems likely that this high incidence of pituitary tumor in the experimental rats is due to metabolites derived from the mold.

INTRODUCTION

Saxton *et al.* (1) reported chromophobe adenoma-like lesion in 92 of 362 pituitaries of Yale strain rats. Thompson *et al.* (2) observed five adenomas of pituitaries in albino Sprague-Dawley rats 19 to 30 months of age. Kim *et al.* (3) found that pituitary tumors were the most common neoplasma in over 27% of rats surviving beyond 17 months of age. Ito *et al.* reported that 28 adenomas spontaneously occurred in 40 of the Charles River strain over 640 days old (4). Therefore, it is generally accepted that pituitary tumor spontaneously occurs with aging and genetic basis. It has been suggested, however, that environmental factors, especially exposure to mold, might be responsible for the so-called spontaneously occurring pituitary tumors (5). *Aspergillus versicolor* (Vuillemin Tiraboshi) is quite common in the human environment and contaminates various grains such as wheat, corn, and rice. We used germ-free rats monoassociated with this mold, before birth and continually thereafter, to clarify the role of the mold in tumorigenesis in the pituitary gland.

MATERIALS AND METHODS

Germ-free Wistar rats produced by our own technique through more than 40 generations in our laboratory (6) were maintained in plastic isolators. Their germ-free status was checked as described by Wagner (7). The diet was of home-made pellets whose composition was reported previously (6). A test tube with a rubber cap in which *A. versicolor* was cultured on Sabouraud dextrose agar was introduced into an autoclave attached to the germ-free isolator, and its surface was sterilized under peracetic acid spray. Then the tube was transferred into the germ-free isolator and the pellet diet therein was contaminated with *A. versicolor* alone (8). As time passed after this monocontamination, the mold not only grew on the diet but spread all over the isolator. Colonies were observed even on the inner surface of the isolator's plastic film. Under this condition, the monoassociated females gave birth to offspring, thereby monoassociating the offspring with the mold or its metabolites prior to birth. Fifty-three of these offspring (the first generation) exposed during gestation were used as experimental rats, while 41 germ-free rats were used as untreated controls.

All the experimental and control rats were maintained in air-conditioned isolators, with 12-h light and dark cycles at a temperature 22 ± 1°C, for 2 years. The experimental rats were exposed to *A. versicolor* not only during gestation but also throughout the entire course of this study. Rats that were either found dead or killed when moribund during the period of observation, or which were killed at the termination of the study, were autopsied for examination. Germ-free control rats were killed on day 730. Live rats were sacrificed under ether anesthesia.

Tissue samples were taken of the endocrine organs, liver, lung, heart, and kidney. Any other unusual-appearing tissue was also sectioned for examination. For light microscopy, tissues were generally fixed in 10% formalin or Carnoy's fluid and embedded in paraffin. Embedded tissues of the endocrine organs were serially sectioned. Those of other organs were randomly sectioned. Sections were stained with H&E for histological examination, and by the chromic acid-Schiff method for mold. Enzyme immunohistochemistry was employed to examine hormone immunoreactivity in the tumor cells of the pituitary. Immunohistochemical staining was carried out according to the indirect method of Nakane (9). Rabbit antisera to GH,[3] LTH, TSH, ACTH, LH, and FSH were purchased from DAKO Corporation, CA. The immunological cross-reactions of the rabbit antisera to GH, LTH, TSH, ACTH, LH, and FSH with these hormones of the rat have been reported (9, 10). We confirmed that the immunohistochemical staining for pituitary hormones in normal control tissue demonstrated a pattern similar to that reported previously.

Parenthetically, it has been confirmed that the *A. versicolor* used in this experiment produces sterigmatocystin and versicolorin A, B, and C (8).

RESULTS

The incidence of tumors in germ-free rats (540–730 days in age, both sexes) exposed to *A. versicolor* or its metabolites before birth and continually thereafter, and in control germ-free rats (730 days) are shown in Table 1. Thirty-three of 53 monoassociated germ-free rats had pituitary tumors (62.3%) while only three of 41 control germ-free rats had such tumors (7.3%). There was no neonatal mortality associated with exposure to *A. versicolor*. The first pituitary tumor of the experimental rats was observed in a female rat killed on day 540 when moribund and most of the pituitary tumors were seen at final sacrifice. Thirty of 33 pituitary tumors were observed grossly. Microscopically, tumors composed largely of eosinophilic cells were present in three experimental cases. No largely eosinophilic cell tumors were found in germ-free controls. All other tumors seemed to consist of seemingly chromophobic cells (Figs. 1 and 2). There was some multiplicity of pituitary tumors in the experimental rats. We did not observe multiple tumors grossly, but we histologically observed isolated tubular and hemangiomatous patterns. We had 10 cases of pituitary hyperplasia in the experimental rats. Most of the adenomas were sharply circumscribed, showing compression of surrounding normal tissue, while most of the hyperplasia were not sharply circumscribed. The enzyme-labeled antibody method confirmed that not only the eosinophilic tumor cells but also the chromophobic tumor cells showed immunoreactivity for LTH or LTH and GH, indicating that they were prolactin cell tumors (Table 2 and Figs. 3 and 4). However, TSH, ACTH, LH, and FSH

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[3] The abbreviations used are: GH, growth hormone; LTH, prolactin; TSH, thyroid-stimulating hormone; ACTH, adrenocorticotrophic hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone.

Table 1 *Incidence of pituitary tumors in germ-free rats monoassociated with A. versicolor and in control germ-free rats*

	Rats examined		Rats with tumor (%)	
	Male	Female	Male	Female
Monoassociated germ-free rats	31	22	19 (61.3)	14 (63.6)
Control germ-free rats	21	20	2 (9.5)	1 (5.0)

Table 2 *Immunoperoxidase reaction for LTH and GH in pituitary tumors*

	Rats with tumor	Tumors with immunoreactive	
		LTH (%)	LTH/GH (%)
Monoassociated germ-free rats	33	26 (78.8)	4 (12.1)
Control germ-free rats	3	2 (66.7)	0

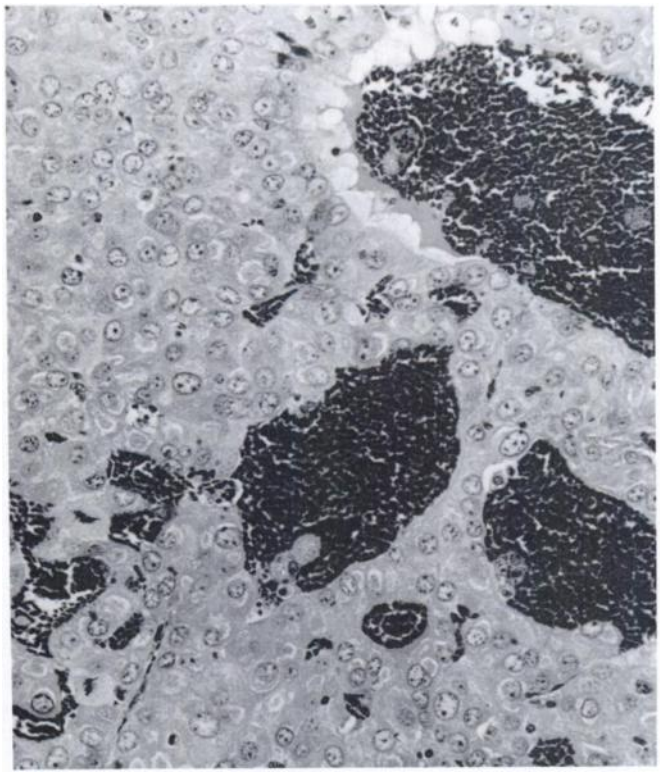

Fig. 1. This pituitary adenoma showed a diffuse pattern. Note dilated blood vessels (H&E, × 250).

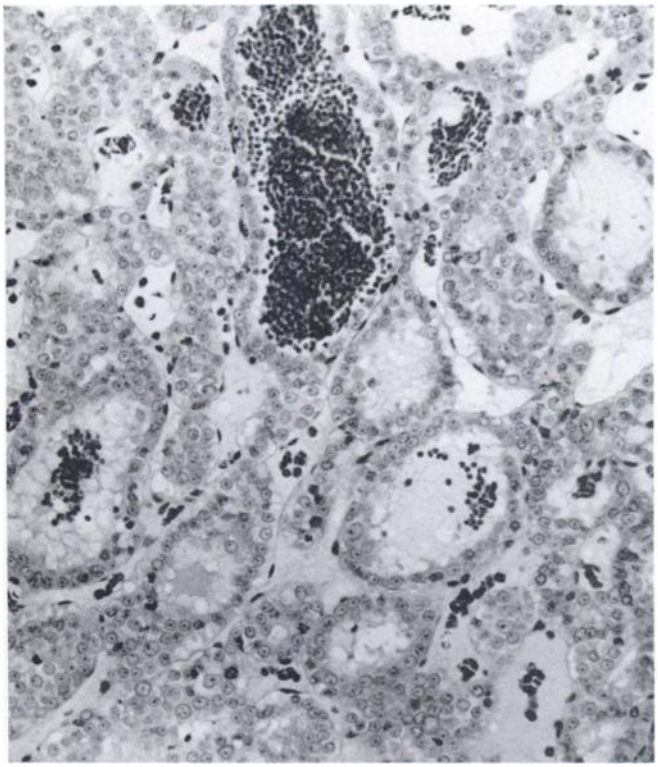

Fig. 2. This pituitary adenoma showed a tubular pattern (H&E, × 250).

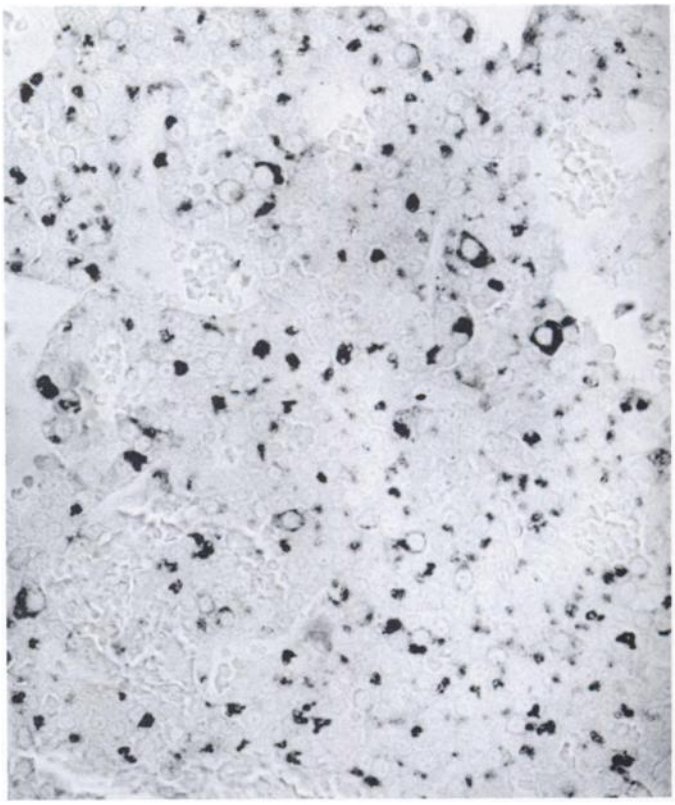

Fig. 3. Immunostaining for prolactin. The majority of tumor cells contain immunoreactive LTH (× 450).

were negative in all specimens examined. The hyperplasia also had positive immunoreactivity for LTH and GH.

In experimental rats, pituitary adenoma was associated in five cases with parathyroid adenoma, three cases with islet cell adenoma, two cases with follicular adenoma of the thyroid, and one case with cortical adenoma of the adrenal. The parathyroid adenoma was the result of cell proliferation and was sharply circumscribed, having a rim of normal parathyroid tissue (Fig. 5). In two experimental cases, pituitary adenoma was associated with both parathyroid and islet cell adenoma. On the other hand, in germ-free controls, none of the pituitary tumors were associated with other endocrine tumors.

One point that deserves special consideration is the fact that 15 (68.2%) of the old female rats monoassociated with *A. versicolor* showed hypertrophy of the uterus and hyperplasia of the mammary gland.

The chromic acid-Schiff staining showed the presence of mold spores in all of the lungs and the presence of mycelium in some of the intestinal tracts of treated cases, indicating mold infection. Lesions other than tumors in the experimental rats were characterized by many granulomas in the lung, nephron destruction in the kidney, necrosis in the liver, and fibrosis in the pancreas.

DISCUSSION

There have been many reports on spontaneously occurring tumors in the pituitary gland of aged animals (2–4, 11). It is

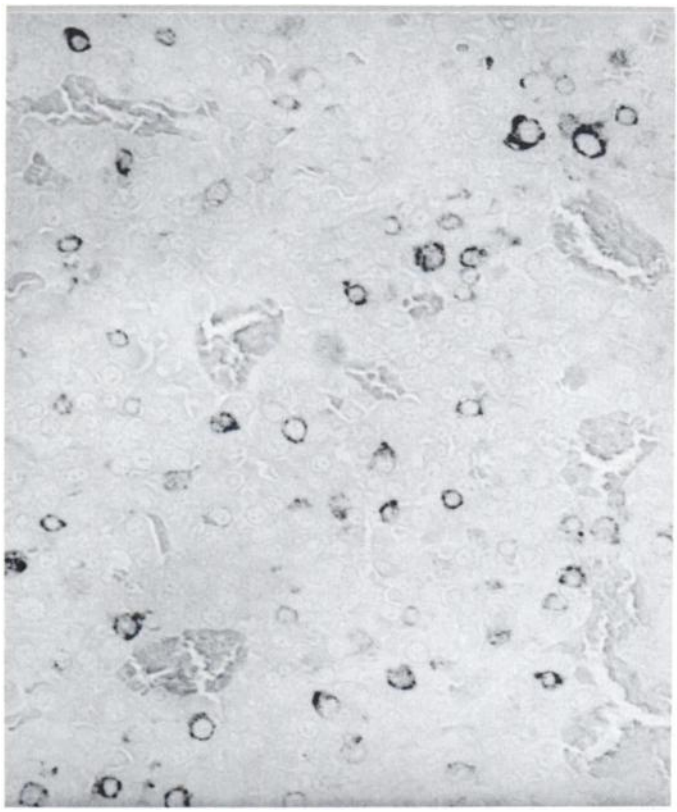

Fig. 4. Immunostaining for growth hormone. Scattered immunoreactive GH cells (× 450).

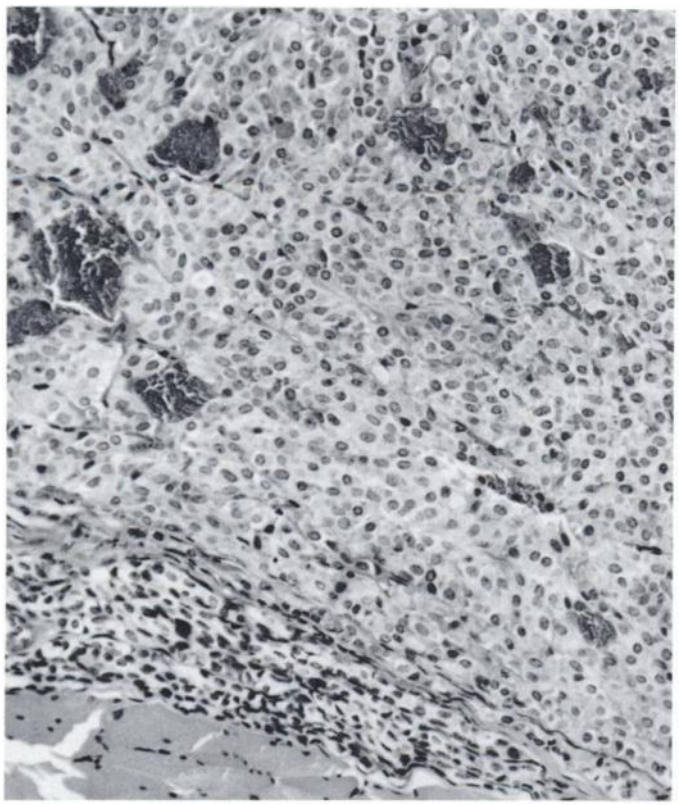

Fig. 5. Parathyroid adenoma with a rim of normal parathyroid tissue (H&E, × 250).

generally accepted that the tumor incidence in this organ increases with age. Pituitary tumors also reportedly occur on a genetic basis, so we must take into account the influence of aging or genetic factors on the development of these tumors. Nevertheless, we cannot exclude the possibility of the environmental influence of mold on tumorigenesis in this organ. Some authors reported that incidence was higher in females, and others reported the contrary. But in our study, there was no significant difference in tumor incidence between males and females.

There was a large wheat crop in the U. S. and a large rice crop in Japan in 1986, and the wheat and rice could have been exposed to weather on account of a shortage of storage space. Such exposure encourages mold contamination. The action of various metabolites derived from mold is an important problem. Schoental *et al.* (5) reported that *Fusarium* mycotoxin may be involved in the occurrence of spontaneous tumors when present in animal diet as a result of occasional fungal growth. Interestingly, estrogenic effect of mold exposure, such as hypertrophy of the uterus and hyperplasia of the mammary gland (12, 13), account for the fact that estrogen treatment caused pituitary tumor, especially prolactin cell tumor (14–16). We cannot exclude the possibility that this mold could play some role as a promoter of pituitary carcinogenesis, particularly considering the ubiquitous natural pervasiveness of mold toxins. Hepatic tumor was produced by administration of sterigmatocystin, which is a metabolite of *A. versicolor* (17), but we had no such tumors in this experiment.

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