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Transplantation of ACTH-Secreting Pituitary Tumor Cells in Athymic Nude Mice *

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Summary. Chronic excess of glucocorticoids results in Cushing's syndrome in humans. A common cause of excess cortisol secretion is the presence of an adrenocorticotropin secreting pituitary tumor which stimulates the adrenal cortex to produce excess glucocorticoids. ACTH-secreting AtT-20 mouse pituitary cells transplanted subcutaneously in oestrogenized athymic nude mice form tumors rapidly. Six weeks after receiving the tumor transplants, the mice weighed 45% more than normal mice due to the increase in body fat. The tumor-bearing mice exhibit the familiar "buffalo hump" appearance due to the abnormal distribution of body fat. The adrenal glands of the tumor-bearing animals are enlarged due to hypertrophy of the zona fasciculata. The foamy looking fasciculata cells in normal mice were converted to dense, eosinophilic cells in the tumor-bearing mice. Transplantation of normal pituitary glands to athymic nude mice with or without oestrogen treatment did not produce these morphological changes. The experimental model described here may be useful for future studies of Cushing's syndrome.

Key words: Athymic nude mice – ACTH pituitary tumor – Cushing's syndrome – Adrenal – Glucocorticoids

The clinical and metabolic abnormalities associated with elevated cortisol secretion were described by Cushing 50 years ago (Cushing 1932). A common cause of excess cortisol secretion is the presence of an adrenocorticotropin (ACTH) secreting pituitary tumor which stimulates the adrenal cortex to produce an excess of glucocorticoids (Liddle 1980).

Cushing's syndrome in the dog (Capen and Martin 1975) and horse (Moore et al. 1979) has been reported. A model for Cushing's syndrome in mice was described by Furth (1955), who

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transplanted ACTH secreting pituitary tumors from mice of the strain LAF_1. The tumor was grafted into the thigh muscle of the animal and palpable tumors developed after a long period of latency (varying from 110 to 207 days). An ACTH-secreting tumor cell line (AtT-20) was subsequently established in vitro from this transplantable pituitary tumor (Buonassisi et al. 1962). The AtT-20 cells were shown to produce biologically active ACTH (Eipper and Mains 1980; Gumbiner and Kelly 1981; Orth et al. 1973). These cells were also widely used to study the biosynthesis of ACTH (Mains and Eipper 1976).

Athymic nude mice, because of their deficiency in immune response, have been commonly used as graft recipients. Many tumor cells have been successfully transplanted into the nude mice (Fogh et al. 1977; Giovanella et al. 1974).

We describe here the morphological changes in athymic mice which had received a subcutaneous transplant of ACTH-secreting AtT-20 cells and the usefulness of these mice as an animal model of Cushing's syndrome.

Materials and Methods

Cell Culture

The AtT-20 mouse pituitary tumor cell line was obtained from the American Type Culture Collection, Rockville, MD., USA. Stock cultures of AtT-20 cells were maintained in T-75 flasks with Dulbecco's modified Eagle's medium supplemented with L-glutamine (4 mM), glucose (4.5 g/l), penicillin (100 IU/ml), streptomycin (100 µg/ml), bovine insulin (10 µg/ml) and 10% (v/v) fetal bovine serum. Cells were kept in a humidified atmosphere of 95% air – 5% CO_2 at 37° C.

Animals

Four to five week old female Balb/c athymic nude mice were obtained from ARS/Sprague Dawley Division, Madison, WI, USA. Animals were kept under standard conditions for a 5 to 7 day period prior to use. The animals were kept inside a laminar-flow air filtration system, and food and water were supplied ad libitum.

Inoculation of Cells in Nude Mice

Cells were detached with Trypsin-EDTA in Hank's balanced salt solution and resuspended in a small volume of medium. AtT-20 cells (10^6) were injected subcutaneously in the flanks of the animals. Some animals also received 500 µg oestradiol valerate once every 2 weeks. Oestrogen was injected subcutaneously in the dorsal midline, caudal to the neck. Pituitaries from normal female Sprague Dawley rats were transplanted subcutaneously into some of the athymic nude mice. Two pituitaries were transplanted into each nude mouse.

Four Groups of Mice Were Studied. Group 1 animals were injected with oestrogen and AtT-20 cells; group 2 animals received oestrogen only; group 3 animals received both oestrogen and pituitary transplants; group 4 animals received pituitary transplants only.

Histology of the Adrenals of the Nude Mice

At the completion of the experiments, the adrenals and whole pituitary glands from the various groups of animals were dissected and weighed. The adrenals were fixed in 3% paraformaldehyde and prepared for histological examination. Sections were stained by haematoxylin and eosin.

Results

The AtT-20 cells form tumors rapidly after transplantation into athymic nude mice. Palpable tumors can be observed 2 to 3 weeks after transplantation. The histology of the AtT-20 tumor is shown in Fig. 6.

The appearance of an oestrogenized normal athymic nude mouse and that

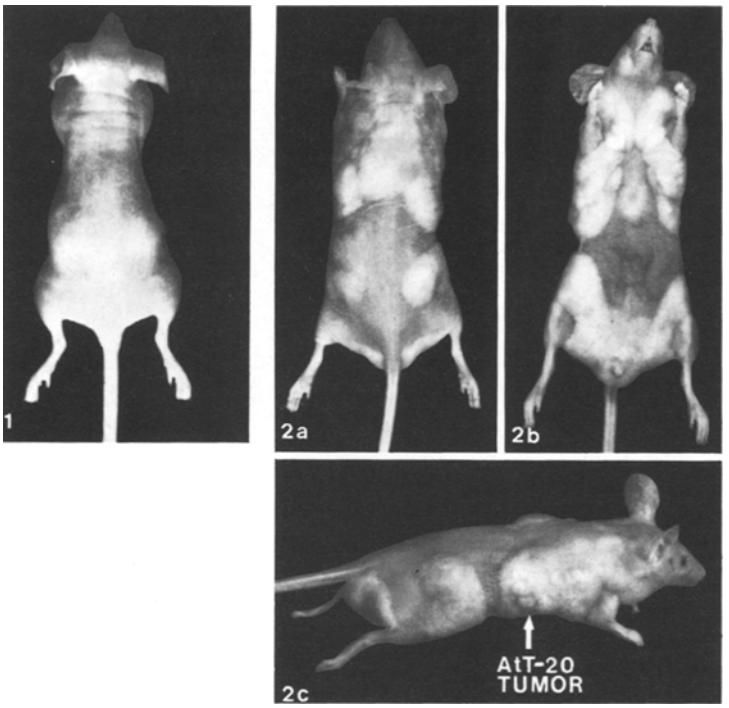

Fig. 1. Control athymic nude mouse. Injected with estrogen only

Fig. 2. a AtT-20 tumor-bearing mouse (dorsal view). Fat deposit around neck and scapular region **b** AtT-20 tumor-bearing mouse (ventral view). Fat deposit around pectoral region and pelvic region. Abdominal area is devoid of any noticeable fat deposit. **c** AtT-20 tumor-bearing mouse (lateral view) exhibiting the "bufallo hump" appearance. The skin is thinner than the control animals

Table 1. Comparison of body weight of AtT-20 tumor-bearing[a] and control athymic nude mice

Animal group ($n=4$)	Body weight (g)	
	Initial	Final[b]
(1) Estrogen + AtT-20 tumor	19.7 ± 0.7	32.4 ± 3.1
(2) Estrogen only	19.5 ± 0.5	22.3 ± 1.5
(3) Estrogen + pituitary transplant	21.3 ± 1.4	23.5 ± 2.0
(4) Pituitary transplant only	19.6 ± 1.3	23.4 ± 2.1
P (group 1 vs other groups)	N.S.	< 0.05

Each value represents the mean $\pm$ S.D.

[a] 10^6 AtT-20 mouse pituitary tumor cells were injected subcutaneously into animals in group (1)

[b] The final measurements were performed 56 days after the initiation of treatment

of an oestrogenized AtT-20 tumor-bearing mouse are shown in Figs. 1 and 2. The pattern of body fat deposits of the tumor-bearing animals (Fig. 2a–c) (group 1) was strikingly different from the control mice (group 2) (Fig. 1). In the former, deposits were distributed at the interscapular and the pelvic regions and along the milk lines. The newly deposited fat was primarily white fat.

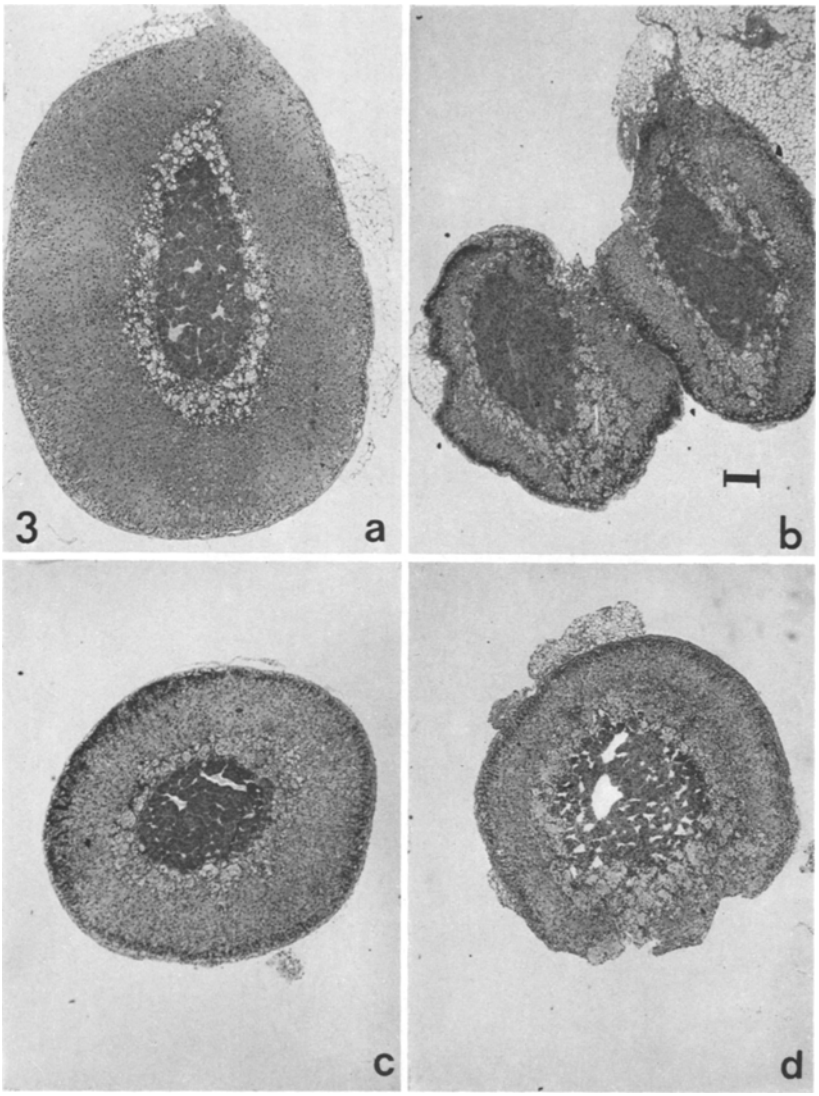

Fig. 3a–d. Plastic sections (2 μm) of adrenal glands from 4 groups of mice. **a** oestrogen treated plus AtT-20 tumor; **b** oestrogen-treated only; **c** with normal rat pituitary transplant; **d** oestrogen treated, and with normal rat pituitary transplant. Each section (hematoxylin and eosin staining) represents the approximate midpoint of a series of sections cut through the whole adrenal gland (in **b**, both halves of adrenals are included). *Scale bar* (in b) represents 200 μm. Magnification ×23 in all figures

The skin of the tumor-bearing mice was thinner than that of control mice. In addition, the mammary glands of the tumor-bearing mice contained milk-like secretion (Fig. 7), an observation similar to that reported by Furth (1953) in the LAF_1 mice. Transplantation of non-ACTH secreting pituitary tumor cells (GH_3) (Leung and Shiu 1981) or of normal rat pituitary glands into the nude

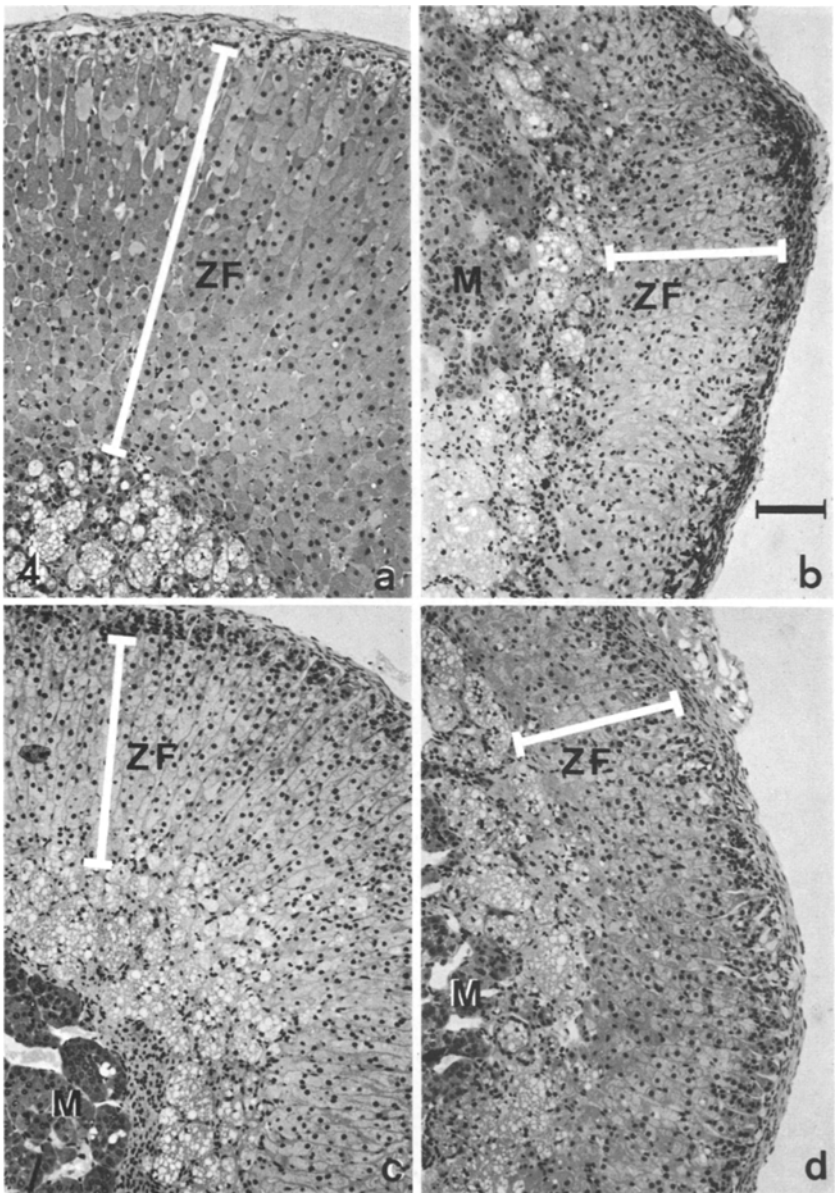

Fig. 4a–d. Enlargement of sections in Fig. 3 from same 4 groups of mice. The zona fasciculata (*ZF, white bar*) of the adrenal cortex consists of rows of eosinophilic cells, which appear enlarged in the mouse bearing AtT-20 tumor (**a**). The zona glomerulosa in **a** appears compressed against the outer capsule. Part of the innermost cortical area, the zona reticularis, is shown at bottom of (**a**) and so the medulla is outside the field of the photograph. In the other groups (**b, c** and **d** as for Fig. 3), the medulla (*M*) is included, as well as all 3 cortical zones (*ZF, zona fasciculata*). Magnification ×92; *scale bar* in (**b**) represents 100 μm

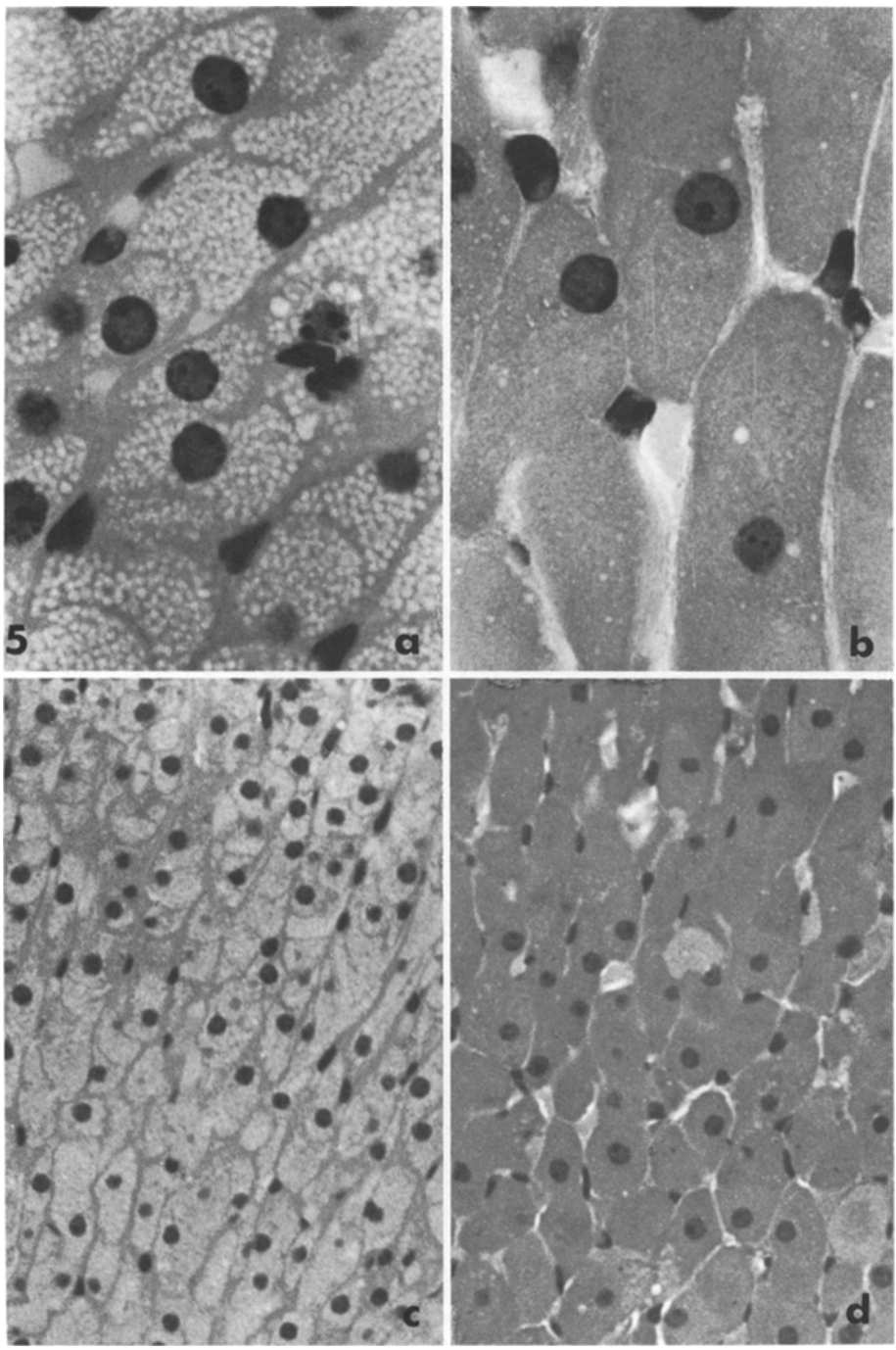

Fig. 5. Fasciculata cells in control and AtT-20 tumor-bearing athymic nude mice. The fasciculata cells of the controls have a foamy cytoplasm filled with lipid vesicles (**a** and **c**). The fasciculata cells of groups 2, 3 and 4 are similar to one another in histological appearanc. In contrast, the fasciculata cells of the AtT-20 tumor-bearing animals are eosinophilic and dense (**b, d**). Magnification for (**a** and **b**) is $\times 1{,}080$; (**c** and **d**) is $\times 332$

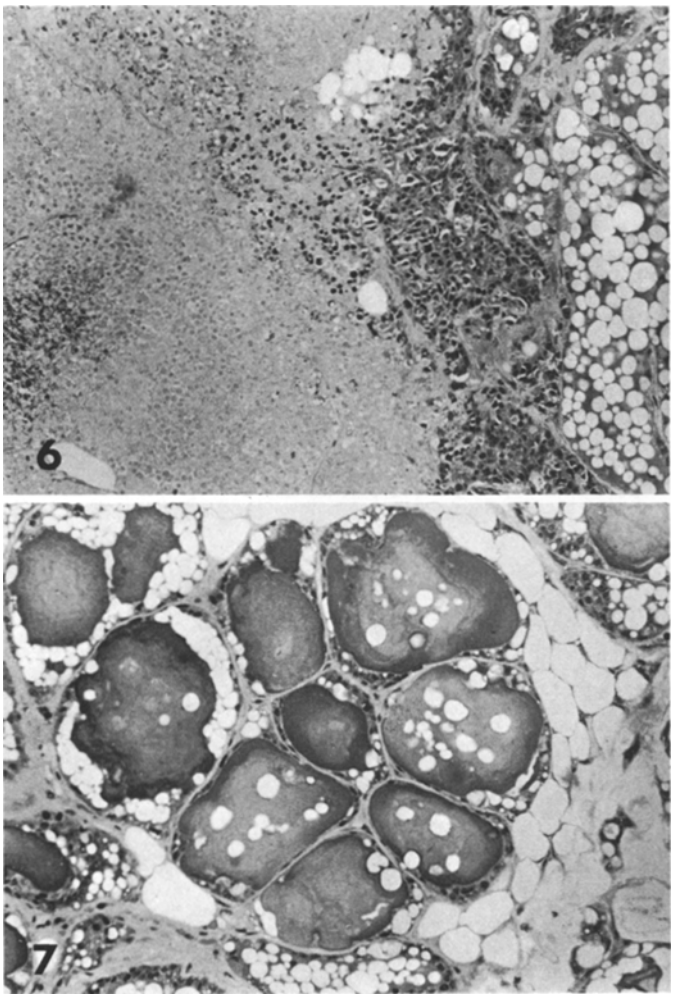

Fig. 6. Histological appearance of the AtT-20 tumor. The AtT-20 tumor is composed of small epithelial cells surrounded by host vascular and adipose tissue. Magnification $\times 120$

Fig. 7. Mammary gland of AtT-20 tumor-bearing athymic nude mice. The mammary glands of the tumor-bearing animals are filled with milk-like secretions. Mammary glands from control nude mice (not shown) are free of secretions. Magnification $\times 120$

mice with oestrogen (group 3) or without oestrogen (group 4) did not produce the changes observed for AtT-20 tumor-bearing animals.

The final mean body weight of the AtT-20 tumor-bearing mice (group 1) was 45% greater than that of mice in the controls (groups 2, 3, 4), a statistically significant difference ($P < 0.05$) (Table 1).

The pituitaries of AtT-20 tumor-bearing mice (group 1) and control animals (group 2) were dissected and weighed after the completion of the experiment on day 56. No significant changes in gross morphology or in weight

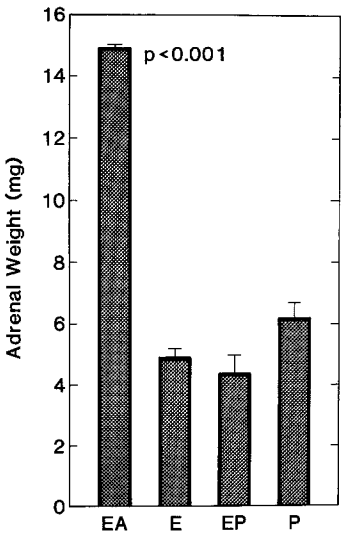

Fig. 8. Adrenal weight of AtT-20 tumor-bearing and control athymic nude mice. The weight indicates the mean of the combined weights of both adrenals of each mouse 56 days after treatment. Each value represents the mean ± S.D. *EA*, oestrogen plus AtT-20 tumor; *E*, oestrogen; *EP*, oestrogen plus pituitary transplants; *P*, pituitary transplants only

(1.22 ± 0.03 mg vs. 1.27 ± 0.07 mg for group 1 and group 2, respectively) were observed.

The adrenals of the AtT-20 tumor-bearing mice were heavier than those of the other gorups ($P < 0.001$) (Fig. 8). The adrenals of mice with pituitary transplant only (group 4) (6.2 ± 0.5 mg) were slightly heavier than the adrenals of animals in group 2 and 3 (4.9 ± 0.3, 4.4 ± 0.6 mg, respectively). This may be due to growth inhibitory effect of oestrogen. Histologically, the adrenals of the tumor-bearing mice (Fig. 3a) consisted of an enlarged cortical layer when compared with the adrenals of mice not bearing AtT-20 cells (Fig. 3b–d). The enlarged adrenal cortex of the AtT-20 tumor-bearing animals was a result of apparent hypertrophy and hyperplasia of the cells of the zona fasciculata, shown in Fig. 4a at higher magnification. The fasciculata cells are known to produce glucocorticoids. The adrenals of mice given only oestrogen (Figs. 3b and 4b) and of mice with transplanted rat pituitary glands with (Figs. 3d and 4d) or without oestrogen treatment (Figs. 3c and 4c) were similar in histological appearance.

The cytoplasm of the fasciculata cells of the control animals showed a foamy appearance, a result of lipid droplets stored in the cells (Fig. 5a and c). In contrast, the fasciculata cells of the adrenals of the tumor-bearing mice had dense eosinophilic cytoplasm (Fig. 5b and d).

Discussion

It is well known that certain tumors may produce symptoms by means of production of humoral and hormonal factors. This phenomenon has been called the ectopic hormone syndrome. The association of Cushing's syndrome with carcinoma is one of the most common ectopic hormonal syndromes. It was found that in patients with Cushing's syndrome associated with a cancer, the

primary tumor and its metastases secreted large amounts of ACTH (Liddle et al. 1969).

Patients with Cushing's syndrome are protein depleted as a result of excess protein catabolism. The skin and subcutaneous tissues are thin and the muscles are poorly developed. Body fat is redistributed in a characteristic way. The extremities are thin, but fat collects in the abdominal wall, face and upper back. As the thin skin of the abdomen is stretched by the increased subcutaneous fat depots, the subdermal tissues rupture to form reddish-purple striae.

Since AtT-20 cells are known to secrete ACTH, it is of interest to study the effects of these cells in vivo. The transplanted AtT-20 mouse pituitary tumor cells formed tumors rapidly in the oestrogenized athymic nude mice. We injected oestrogen into the nude mice because we had observed previously that oestrogen stimulated the growth of other pituitary tumor cells (Leung and Shiu 1981). Interestingly, the growth of the AtT-20 cells was also stimulated by oestrogen in vivo suggesting that steroid hormones may be involved in the aetiology of these pituitary tumors. AtT-20 cells also form tumors in athymic nude mice without oestrogen treatment, but with a longer latent period and a slower growth rate (data not shown).

The adrenals of the AtT-20 tumor-bearing mice were significantly heavier than the controls. The zona fasciculata became hyperplastic while the zona glomerulosa was not affected. These observations are similar to clinical findings in humans. The fasciculata cells of the tumor-bearing animals became dense and eosinophilic when compared with the lipid laden fasciculata cells of the controls. This may be due to an increase in glucocorticoid production and secretion by the adrenal cortex.

The AtT-20 tumor-bearing animals exhibited a pronounced phenotypic change with increase in body weight and altered distribution of body fat. The interscapular fat deposit clearly seen in these animals resembled the "buffalo hump" appearance described in patients with Cushing's syndrome. The AtT-20 tumor-bearing animals also have an increased appetite and intake of food, although food intake was not quantitated in this study. Since AtT-20 tumor cells were known to secrete, as well as ACTH, such other peptides as β-lipotropin and β-endorphin (Gumbiner and Kelly 1981), the behavioral changes of these tumor-bearing mice may be related to the actions of these neuropeptides.

The experimental model described in this report provides a reproducible system for studying Cushing's syndrome. Both the genetic background of the athymic nude mice and the number of AtT-20 cells injected can be standardized. The AtT-20 cells readily form ACTH-secreting tumors in the nude mice, approximately 2 weeks after inoculation. In contrast, a long latent period was required when the ACTH-secreting tumors were transplanted into LAF_1 mice (Furth 1955). The small size of the nude mice make them easy to keep and handle. These animals can be maintained in a pathogen-limited environment (by the use of filter bonnets or filtered air). Another advantage is that unlike other models which used fur-bearing animals (such as dogs), the athymic nude mice do not have fur. Thus, the distribution and enlargement of the fat deposits can be readily observed and monitored. This will allow the investigators to study the various stages of the development of the disease. Future studies on

the biochemical changes of these animals transplanted with ACTH-secreting tumors may provide some interesting insight into the disease.

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