The Metabolism and Binding of Testosterone in Androgen-dependent and Autonomous Transplantable Mouse Mammary Tumors

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SUMMARY

The localization of androgens in two lines of transplantable mouse mammary tumors, one androgen dependent and the other autonomous, has been studied after the i.v. administration of 200 µCi (1.4 µg) of Δ4-androsten-17β-ol-3-one-1,2-3H to tumor-bearing mice. Whereas at 30 min the uptake of radioactivity was approximately equal in intact, dependent, and autonomous tumors, the level of radioactivity was almost 4 times higher in nuclei of dependent cells than in nuclei of autonomous cells. Metabolites of Δ4-androsten-17β-ol-3-one-1,2-3H were identified by thin-layer and gas-liquid chromatography, and no appreciable differences were found in the intracellular concentrations of Δ4-androsten-17β-ol-3-one and 5α-androstan-17β-ol-3-one. Steroid-binding protein was precipitated from the 105,000 x g supernatant fraction of cytoplasm (cytosol) with ammonium sulfate, and binding was measured by gel filtration. The amount of steroid bound to the receptors was 4 times higher in cytosol of dependent cells than in cytosol of autonomous cells, and there was a corresponding difference in the specific activities of the binding proteins. These results are consistent with the view that the impaired incorporation of androgens into nuclei of autonomous cells is related to a reduced concentration of cytoplasmic receptors.

INTRODUCTION

Target tissues that are resistant to the effects of steroid hormones frequently differ from their sensitive counterparts in 2 respects. First, the capacity of cytoplasm from resistant cells to bind steroids is reduced, and, secondly, the incorporation of steroids into nuclei is relatively diminished. If not both, one or the other of these changes has been demonstrated in cases of rat mammary carcinoma induced with 7,12-dimethylbenz(a)anthracene, which does not regress after oophorectomy (21, 33, 36, 43); of the transplantable rat mammary carcinoma R3230AC (32); of the pregnancy-induced mammary carcinoma of GR/A mice (48); and of certain other steroid-insensitive murine breast cancers (46). Similarly, the autonomy of primary carcinoma of the human breast has been tentatively linked to the absence of estrogen binding in cytoplasm (21); the resistance of lymphoma cells to glucocorticoids is thought to be related to a reduction in binding capacity at the cytoplasmic and nuclear levels (1, 20, 24, 25, 40) and the degree of induction of tyrosine aminotransferase appears to be proportional to the binding of steroid in cytoplasm (2, 3, 28, 42). In the genetically determined condition of testicular feminization affecting rats, mice, and humans, it has been proposed that the failure of target organ development is causally related to decreased intracellular binding of testosterone and dihydrotestosterone (7, 8, 16, 17, 37). Together these observations clearly strengthen the concept that certain aspects of the response of target cells are regulated by steroid-protein complexes that are formed in the cytoplasm and migrate into the nucleus (22, 51).

In spite of the attractiveness of this hypothesis, there is growing evidence that tends to offset the presumed correlation between the degree of sensitivity of a tissue to steroid hormones and the concentration of receptors. First, no relationship has been found between the estradiol-binding capacity and estrogen responsiveness of dependent and autonomous hamster kidney tumors (47). Second, some doubt has arisen as to whether the autonomy of the R3230AC rat mammary carcinoma can indeed be attributed to the lack of estradiol-binding protein (49). Third, a significant number of human mammary carcinomas in which receptors can be demonstrated fail to respond to endocrine therapy (21). Fourth, while the presence of estrogen receptors in human mammary cancers is certain, proof of the existence of receptors in normal breast and benign tumors of the breast is less convincing (13, 14, 19, 23, 26, 50). Finally, the identification of a nuclear receptor for dihydrotestosterone in kidney cells of mice with the Tfm mutation (9) is clearly inconsistent with the idea that the resistance of target tissues in this variant of testicular feminization is related to the absence of intracellular

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1 The project was financed by grants from the Medical Research Council of Canada (MA 3729), the National Cancer Institute of Canada, and the Ontario Cancer Treatment and Research Foundation.

2 Scholar of the Medical Research Council of Canada.

Received December 14, 1972; accepted March 21, 1973.
Cytosol was prepared from the 400 x g supernatant through centrifugation at 11,000 x g for 30 min in a Sorvall RC-2B refrigerated centrifuge (SS-34 rotor, rav 8.3 cm) and resuspended in 1 ml of Tris-HCl buffer, pH 7.0, containing EDTA (0.05 mM), MgCl₂ (5 mM), mercaptoethanol (0.5 mM), and NaCl (50 mM), in preparation for gel-filtration chromatography.

MATERIALS AND METHODS

Experimental Tumors. The SC-115 androgen-dependent mouse mammary carcinoma was obtained from Dr. T. Minesita of the Aburahi Laboratories, Koko-cho, Japan, and has been maintained by serial transplantation in DD/S mice both in Edmonton and in Toronto. Transfer of the androgen-dependent cells to female mice occasionally results in growth of a tumor. After 1 or 2 passages in female mice, the proliferative capacity of such a tumor is lost except in rare instances. One tumor has survived through several generations and grows equally well in intact males, castrated males, and females. Hence, this line of cells is believed to be autonomous as compared to the androgen-dependent line which grows only in intact males. Histologically, the dependent tumor is composed of sheets of anaplastic cells supported by delicate fibrous trabeculae. The autonomous tumor is similar in appearance.

Homogenization of Tissue. Tumors weighing 1 to 15 g were dissected free of s.c. tissue and minced as described previously (5). Following homogenization of the tissue fragments in a Dounce apparatus, centrifugation of the homogenate at 400 x g for 10 min yielded crude cytoplasmic and nuclear fractions (5). The nuclei were further homogenized in Solutions A and B as described in the method of Magliozzi et al. (30). The Chauveau step was omitted; instead the crude nuclear pellet was centrifuged through a discontinuous sucrose gradient. Details of the latter procedure are available elsewhere (5). Extracts obtained by this method were subjected to 2 chromatography steps: in Step 1 a silicic acid column (1 x 16 cm) was used to remove polar compounds and lipids; in Step 2 a silicic acid column (3 x 70 mm) was used to remove polar compounds and cholesterol (4). Appropriate steroid standards were added to the samples prior to analysis by thin-layer and gas-liquid chromatography.

Chromatography of Steroids. Steroids in purified tissue extracts were identified as previously described (5) by a combination of thin-layer chromatography on aluminum oxide and gas-liquid chromatography. As well, in some experiments, acetylated derivatives were formed and thin-layer chromatography was used to identify the products. Since the results obtained by these methods were similar, estimation of the relative amounts of the principal intracellular steroids was subsequently accomplished by thin-layer chromatography alone.

Radioactive Materials. Testosterone-1,2-3H (5 mCi/0.0282 mg) was purchased from New England Nuclear (Boston, Mass.). Purity was checked by thin-layer and gas-liquid chromatography and varied between 93 and 95% in several tests. Solutions for i.v. injections were prepared as follows. To each 200 μl of benzene:ethanol solution containing radioactive steroid were added 20 μl of 5% (v/v) polyoxyethylene sorbitan monopalmitate, and the solution was dried under N₂. The dry preparation was then dissolved in a suitable amount of water containing 5% (v/v) ethanol. Each mouse was given an injection of 250 μl of such a solution.

Liquid Scintillation Counting. Liquid scintillation counting was carried out with either a PPO:toluene solution (4 g of PPO per liter of toluene) or a PPO:toluene solution that contained toluene (1 liter), PPO (6 g), water (75 ml), and Bio-Solv (BBS-3; 116 g; Beckman Instruments, Scientific Instruments Division, Fullerton, Calif.). Samples were deposited in glass vials and the radioactivity was measured in an automatic liquid scintillation system. External stan-
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Protein Determinations. Proteins were measured by the method of Lowry et al. (29), using bovine serum albumin as the reference material.

RESULTS

Incorporation of Radioactivity into Intact Tumors after the Administration of Testosterone-1,2-3H. The incorporation of radioactive androgens was compared in autonomous and dependent tumors at various times from 5 to 60 min after the i.v. injection of 200 µCi of testosterone-1,2-3H into tumor-bearing mice. As shown in Chart 1, the radioactivity rapidly increased to a peak level within 15 min, reaching $27.0 \times 10^5$ dpm/g in the autonomous tumor and $35.0 \times 10^5$ dpm/g in the dependent tumor. Thereafter, the level of radioactivity declined at approximately the same rate in both types of tumor tissue. Any differences in the levels are probably not significant as indicated by the data presented in Table 1, Column 1. When the radioactivity was compared in Dm, Am, and Af tumors 30 min after the injection of testosterone-1,2-3H, all tumors had incorporated similar amounts of radioactivity. Comparative studies were next carried out on nuclei.

Table 1

<table>
<thead>
<tr>
<th>Type of tumor</th>
<th>Radioactivity recovered in tumor (dpm/g, wet wt. $\times 10^{-5}$)</th>
<th>Radioactivity recovered in nuclei (dpm/nucleus $\times 10^4$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dm</td>
<td>$30.3 \pm 4.0^*$ (6)</td>
<td>$8.6 \pm 0.6$ (9)</td>
</tr>
<tr>
<td>Am</td>
<td>$31.0 \pm 3.1$ (17)</td>
<td>$2.3 \pm 0.3$ (19)</td>
</tr>
<tr>
<td>Af</td>
<td>$35.3 \pm 3.1$ (13)</td>
<td>$2.3 \pm 0.2$ (11)</td>
</tr>
</tbody>
</table>

* Mean ± S.E.
* Numbers in parentheses, number of separate experiments performed to obtain the values listed.

Incorporation of Radioactivity into Nuclei after the Administration of Testosterone-1,2-3H. Nuclei were isolated from dependent and autonomous tumors at intervals 5 to 60 min after the injection of testosterone-1,2-3H, and the amount of radioactivity incorporated was measured. As shown in Chart 2, the level of radioactivity in nuclei of dependent tumors increased from a mean of $1.3 \times 10^{-4}$ dpm/nucleus at 5 min to a mean of $9.3 \times 10^{-4}$ dpm/nucleus at 45 min. By contrast, the level of radioactivity in nuclei of autonomous tumors was almost constant and did not exceed the mean value of $1.6 \times 10^{-4}$ dpm/nucleus attained at 15 min. Additional experiments were carried out to confirm the marked disparity in the incorporation of radioactive andro-
gends by nuclei; the results are presented in Table 1, Column 2. At 30 min after the injection of testosterone-1,2,3H, the level of radioactivity in Dm nuclei was 8.6 × 10^4 dpm/nucleus, almost 4 times greater than the level of 2.3 × 10^4 dpm/nucleus in Am and Af nuclei. Thus it is clear that, whereas dependent and autonomous cells cannot be distinguished on the basis of differences in androgen uptake by whole cells, the distinction can be made accurately on the basis of differences in androgen uptake by nuclei.

Recovery of Neutral Steroids. A comparison of the recovery of the neutral metabolites of testosterone-1,2,3H was made at each step of the procedure for purifying intracellular androgens. Thus, 30 min after the injection of 200 µCi testosterone-1,2,3H, cytoplasmic and nuclear samples were extracted with chloroform:methanol and the extracts were chromatographed twice on silicic acid. After each step the recovery of radioactivity was calculated as a percentage of the radioactivity available as starting material before the purification step. The percentage recovery of radioactivity in the chloroform:methanol extract and after the 1st and 2nd chromatographs is shown in Table 2, Columns 1, 2, and 3, respectively. Three points can be drawn from these data. First, except for the greater recovery of radioactivity from the Dm sample after Chromatography 2, no significant difference was found among the Dm, Am, and Af cytoplasmic and nuclear samples at any stage of purification, suggesting that the amount of polar metabolites formed in each type of tumor cell is similar. Secondly, since the overall percentage of radioactivity recovered from nuclei after the 2nd chromatography (57 to 69%) was 2 to 4 times greater than the percentage recovered from cytoplasm (15 to 32%), it appears that the tumor cells have retained the capacity to exclude polar metabolites from the nucleus. Finally, the similarity in the recovery of radioactivity from Dm, Am, and Af nuclei suggests that the development of autonomy has not been accompanied by a loss of specificity for androgen uptake into nuclei, as would have been suggested by an increase in permeability of the nuclear membrane to polar metabolites in autonomous cells.

Identification of Neutral Metabolites in Purified Extracts of Cytoplasm and Nuclei. The steroid constituents of the extracts obtained after the purification procedure described in the previous section were identified by thin-layer chromatography on aluminum oxide. Results are presented in Table 1; the relative amount of each steroid is expressed as a percentage of the total radioactivity in the final neutral steroid fraction. From Table 1, Column 1, it is clear that testosterone is the chief androgen in cytoplasm and nuclei of Dm, Am, and Af cells. In each type of cell the proportion of testosterone in the nucleus is greater than the proportion of this steroid in the cytoplasm. The results presented in Table 1, Column 2, indicate that dihydrotestosterone contributes about 3% to the total neutral steroids in both the cytoplasmic and nuclear extracts. Two observations merit emphasis in connection with these data. First, the relative amount of dihydrotestosterone in Af cytoplasm (1.7 ± 0.1) is significantly below the amount in Am cytoplasm (3.3 ± 0.6) and in Dm cytoplasm (3.3 ± 0.1) (p < 0.01 in both cases), consistent with the view that estradiol inhibits the enzymatic conversion of testosterone to dihydrotestosterone (12, 18, 27, 44, 45). Secondly, the percentage of dihydrotestosterone in nuclei did not increase over the percentage in cytoplasm as did the relative amount of testosterone. From the results in the remaining columns of Table 1, it is clear that the percentage of androstenedione, androstenedione, and androsterone is greater in cytoplasmic extracts than in nuclear extracts. It seems reasonable to believe that the higher proportion of testosterone in nuclei occurs at the expense of androstanedione, androstenedione, and androsterone, which, for the most part, are excluded from the nucleus.

Intracellular Binding of Androgens. In order to examine the relationship between the decreased incorporation of androgens by nuclei from autonomous cells and the concentration of intracellular receptors, we measured the amount of steroid binding in cytoplasm and nuclei 30 min after the injection of testosterone-1,2,3H. The results are listed in Table 2 both as a percentage of the total radioactivity in the cytosol and in nuclei and as the specific radioactivity of the protein recovered. In 8 experiments with Dm cytoplasm, the mean radioactivity recovered in the cytosol fraction was 800,000 dpm (range, 500,000 to 1,800,000 dpm). A small percentage (2.6 ± 0.3) was bound, and the specific radioactivity of the protein was 2800 ± 300 dpm/mg. The corresponding results obtained in 10 experiments with Am cytosol were 400,000 dpm (range, 100,000 to 1,100,000 dpm) recovered, 0.7 ± 0.1% bound, and a specific radioactivity of 600 ± 100 dpm/mg. Therefore, according to both criteria of binding, approximately 4 times more radioactive steroid was bound in Dm cytoplasm than in Am cytoplasm.

Because the mean radioactivity recovered in the Dm cytosol fraction is 2 times greater than that recovered in the Am cytosol fraction, these results appear to be at variance with the data presented in Table 1 which indicate that the incorporation of radioactivity into whole tumors is equal. Since the results listed in Table 4 have not been corrected.

### Table 2

<table>
<thead>
<tr>
<th>Type of tumor</th>
<th>Recovery (%)</th>
<th>Chromatography 1</th>
<th>Chromatography 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Extract</td>
<td>A. Cytoplasm</td>
<td>B. Nucleus</td>
</tr>
<tr>
<td>Dm</td>
<td>55.2 ± 6.7%</td>
<td>62.2 ± 4.6%</td>
<td>93.2 ± 3.0%</td>
</tr>
<tr>
<td>Am</td>
<td>52.9 ± 3.4%</td>
<td>67.5 ± 7.4%</td>
<td>60.5 ± 9.8%</td>
</tr>
<tr>
<td>Af</td>
<td>57.2 ± 4.0%</td>
<td>51.9 ± 5.6%</td>
<td>51.6 ± 7.7%</td>
</tr>
</tbody>
</table>

* Mean ± S.E.

### Notes
- Numbers in parentheses, number of separate experiments performed to obtain the values listed.

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Table 3
Identification of androgen metabolites in neutral steroid extracts

The extracts of neutral steroids following Chromatography 2 described in Table 2 were analyzed by thin-layer chromatography. The relative amount of each steroid identified is expressed as a percentage of the total radioactivity in the neutral steroid fraction. The radioactivity defined as "other" represents material recovered at the origin and in positions that did not correspond to any of the standards.

<table>
<thead>
<tr>
<th>Identification</th>
<th>Type of Tumor</th>
<th>Dihydrotestosterone</th>
<th>Androstanedione</th>
<th>Androstenedione</th>
<th>Androstene</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Cytoplasm</td>
<td>Dm (4)*</td>
<td>71.8 ± 4.3*</td>
<td>3.3 ± 0.1</td>
<td>3.1 ± 0.6</td>
<td>7.9 ± 1.7</td>
<td>5.7 ± 0.9</td>
</tr>
<tr>
<td>Dm (7)</td>
<td>70.7 ± 2.7</td>
<td>3.3 ± 0.6</td>
<td>1.8 ± 0.5</td>
<td>5.5 ± 0.7</td>
<td>6.1 ± 1.6</td>
<td>12.4 ± 1.3</td>
</tr>
<tr>
<td>Af (9)</td>
<td>69.7 ± 2.2</td>
<td>1.7 ± 0.1</td>
<td>2.1 ± 0.4</td>
<td>9.3 ± 1.2</td>
<td>6.7 ± 0.9</td>
<td>10.5 ± 0.8</td>
</tr>
<tr>
<td>B. Nucleus</td>
<td>Dm (4)</td>
<td>86.3 ± 4.1</td>
<td>3.2 ± 0.4</td>
<td>0.6 ± 0.4</td>
<td>1.0 ± 0.3</td>
<td>1.7 ± 0.5</td>
</tr>
<tr>
<td>Dm (8)</td>
<td>89.3 ± 2.2</td>
<td>2.7 ± 0.2</td>
<td>0.4 ± 0.2</td>
<td>1.0 ± 0.2</td>
<td>1.2 ± 0.2</td>
<td>5.4 ± 1.7</td>
</tr>
<tr>
<td>Af (8)</td>
<td>89.7 ± 1.2</td>
<td>2.2 ± 0.5</td>
<td>0.3 ± 0.0</td>
<td>1.1 ± 0.2</td>
<td>1.6 ± 0.3</td>
<td>5.1 ± 0.7</td>
</tr>
</tbody>
</table>

* Figures in parentheses, number of separate experiments performed to obtain the values listed.  
* Mean ± S.E.

Table 4
Binding of radioactive androgens to cytoplasmic and nuclear protein 15 to 30 min after the i.v. injection of testosterone-1,2-3H

Radioactivity recovered in the void volume after gel filtration of an appropriate sample was measured as a percentage of the total radioactivity recovered in the cytosol and nuclear fractions and also as the specific radioactivity of the proteins eluted in the void volume.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Type of Tumor</th>
<th>Experiments</th>
<th>Total in fraction (dpm × 10^-4)</th>
<th>Protein bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Cytosol</td>
<td>Dm</td>
<td>8</td>
<td>800 (500-1800)*</td>
<td>2.6 ± 0.3*</td>
</tr>
<tr>
<td>Am</td>
<td>10</td>
<td>400 (100-1100)</td>
<td>0.7 ± 0.1</td>
<td>600 ± 100</td>
</tr>
<tr>
<td>B. Nucleus</td>
<td>Dm</td>
<td>8</td>
<td>70 (2-170)</td>
<td>25.7 ± 2.1</td>
</tr>
<tr>
<td>Am</td>
<td>7</td>
<td>20 (10-30)</td>
<td>34.0 ± 2.2</td>
<td>1000 ± 200</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, range of values for the radioactivity recovered in cytosol and nuclear fractions.  
* Mean ± S.E.

for loss of cells during the procedure to remove erythrocytes, the difference is probably not significant. The recovery of protein from Dm cytosol after precipitation with ammonium sulfate at 1800 ± 100 μg/g was somewhat greater than the amount recovered from Am cytosol at 1200 ± 100 μg/g. This result would be expected if fewer cells were recovered in the experiments with Am tumors. On the other hand, the possibility that protein is selectively lost from Am cells during the washing procedure or that the Dm cells are more permeable to plasma steroid-binding protein cannot be discounted.

In Dm and Am nuclei, the amount of binding was almost equal when measured as a percentage of the total radioactivity in the nuclear fraction. However, the specific radioactivity of the nuclear protein was approximately 4 times lower in Am nuclei than in Dm nuclei as might be expected from the 4-fold difference in the incorporation of radioactivity described in Chart 2 and Table 1.

Identification of Androgens Bound to Cytoplasmic Protein. The identity of androgens bound to cytosol protein was established by thin-layer chromatography of samples obtained 30 min after the administration of 200 μCi of testosterone-1,2-3H to tumor-bearing mice. From the results shown in Table 5, it is evident that the steroid composition of the binding protein resembles the steroid constituents in nuclei. The similarity is consistent with the idea that androgens combine with a cytosol protein before being transferred into the nucleus (10, 11, 31, 39).

DISCUSSION

In this series of experiments, we have compared the metabolism and binding of testosterone in androgen-
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Table 5
Identification of steroids bound to cytoplasmic protein from Dm tumors

<table>
<thead>
<tr>
<th>Steroid</th>
<th>% radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone</td>
<td>85</td>
</tr>
<tr>
<td>Dihydrotestosterone</td>
<td>3</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>0</td>
</tr>
<tr>
<td>Androstenolone</td>
<td>3</td>
</tr>
<tr>
<td>Androsterone</td>
<td>1</td>
</tr>
<tr>
<td>Other</td>
<td>8</td>
</tr>
</tbody>
</table>

Table 5

The relative amount of each steroid identified is expressed as a percentage of the total radioactivity in the neutral steroid fraction recovered from the protein peak. Values shown are the means of the results of 2 experiments.

dependent and autonomous lines of neoplastic cells derived from the SC-115 mouse mammary adenocarcinoma. Although it is uncertain whether the autonomous line is derived from the dependent line, there are several reasons to believe that both lines are closely related. First, the histological appearance of each tumor is virtually the same. Secondly, in both tumors the metabolism of testosterone yields a similar pattern of intracellular steroids (Tables 2 and 3). Thirdly, the latter results also indicate that the nuclei of dependent and autonomous cells are equally impervious to neutral steroids such as androstenedione, androstanediol, and androsterone and to polar metabolites. These similarities imply that the dependent and autonomous cells originate from a common progenitor cell. Furthermore, they suggest that autonomy is not related to a defect of androgen metabolism or to a loss of selectivity of the transport system that promotes the uptake of androgens by nuclei.

No difference was found in the amount of radioactivity incorporated into whole tumors after the injection of testosterone-1,2-3H (Table 1). On the other hand, the amount of radioactivity incorporated into nuclei of autonomous cells was only one-fourth the amount incorporated into nuclei of dependent cells (Chart 2; Table 1). Measurement of radioactivity in cytoplasm (data not shown) revealed that the amount of label in this fraction was elevated in direct proportion to the reduction of radioactivity in the nuclear fraction. Therefore, it is unlikely that the decreased incorporation of radioactivity into nuclei resulted from the loss of steroids during the nuclear isolation procedure. Our results thus provide evidence that the transport of androgens across the nuclear membrane is indeed reduced in autonomous cells.

In keeping with the decrease in the amount of androgens in nuclei, there was a corresponding reduction in the amount of binding of androgens to cytosol protein in autonomous cells (Table 4). While the percentage of radioactivity bound in cytosol is low, this percentage is greater if it is expressed in terms of the amount of androgens present in cytoplasm. As can be deduced from the data presented in Tables 2 and 3, only a small percentage of the radioactivity in cytoplasm represents testosterone and dihydrotestosterone, the principal androgens bound to cytosol protein. The possibility that binding and nuclear incorporation are reduced in autonomous cells because of increased amounts of endogenous androgens is unlikely in view of the similarity of the results obtained with autonomous tumors of male and female mice.

Although no definitive meaning can be ascribed to the correlation between the reduction of androgen binding in cytoplasm and the parallel reduction of androgen incorporation into nuclei, this finding is nevertheless consistent with the idea that the transport of androgens across the nuclear membrane is decreased in autonomous cells because of the lack of appropriate carrier proteins.

In conclusion, our evidence supports the concept that the insensitivity of target tissues to steroid hormones is distinguished by a relative paucity of steroid binding in the cytoplasm and a relative lack of steroid incorporation into the nucleus. However, it is evident that some insensitive tissues will be found to contain normal levels of receptors (21, 47, 49). In such cases it is possible that autonomy is related to a phenotypic change that does not involve an effect on intracellular binding.

ACKNOWLEDGMENTS

We express our thanks to Erica Robins, Adrian VanSon, and Dagmar LenaNicka for excellent technical assistance. We also thank Dr. T. Minesita for his generosity in providing the DD/S mice that made this study possible.

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