Analysis of Prolactin and Growth Hormone Production in Hyperplastic and Neoplastic Rat Pituitary Tissues by the Hemolytic Plaque Assay

Ricardo V. Lloyd, Kimberlee Coleman, Kristina Fields, and Veena Nath

Department of Pathology, University of Michigan Medical Center, Ann Arbor, Michigan 48109

ABSTRACT

The reverse hemolytic plaque assay (RHPA) was used to detect hormone release from cultured normal, hyperplastic, and neoplastic rat pituitary cells. Hyperplastic pituitary cells were produced by s.c. diethylstilbestrol (DES) treatment (10 mg in Silastic tubes) for 3, 6, and 9 weeks. Neoplastic pituitary cells from rats with MtT/W15 transplantable tumors treated with DES for 3 weeks were also analyzed. All spots of the same cells were also analyzed by immunocytochemical staining. DES treatment resulted in an increase in prolactin (PRL)-producing cells in hyperplastic pituitaries compared to untreated pituitaries after 9 weeks of treatment by the RHPA (61.2 ± 5.2 (SE) versus 32 ± 3.0)] and by immunocytochemical staining [70.9 ± 2.4 versus 36 ± 1.4]). The percentage of mammosomatotropic cells decreased from 11.3 ± 3.8 to 4.2 ± 2.6% in pituitary cells from these same groups of animals. After 3 weeks of DES treatment in rats with MtT/W15 tumor, there was an increase in growth hormone (GH)-producing cells and a decrease in PRL-producing cells when analyzed by the RHPA (control: percentage of GH, 36.3 ± 6.2; percentage of PRL, 39.0 ± 1.6 versus DES-treated tumors: percentage of GH of 68.2 ± 1.9; and percentage of PRL, 3.2 ± 1.8%). The percentage of mammosomatotropic cells declined from 12.4 ± 2.3 to 0.77 ± 2.4%. A combined procedure of RHPA followed by immunocytochemical staining on the same slides also revealed a decline in mammosomatotropic cells after chronic DES treatment in hyperplastic and neoplastic MtT/W15 tumor cells. These results show that DES has different effects on PRL and GH secretion and storage in hyperplastic pituitary and in the MtT/W15 pituitary tumor cells.

INTRODUCTION

Chronic administration of estrogens is known to cause PRL cell hyperplasia and pituitary tumor development in rats (1-11). Recent experiments by several investigators have shown that estrogens can also inhibit the growth of transplantable rat pituitary tumors such as the MtT/F4 and MtT/W15 (5, 6, 9, 12, 13). Recent studies from our laboratory based on studies with transplantable pituitary tumor tissues (13) and in vitro synthesis of PRL or GH in pituitary tumor cells from DES-treated rats (14) suggested that the inhibition of growth of the M+T/W15 tumor by DES was associated with an increase in GH production and a decrease in the PRL-producing cells (5, 14).

The development of a reverse hemolytic plaque assay for pituitary cells has enabled investigators to analyze hormone production by individual cells (15-25). This technique is extremely useful for studying heterogeneous cell populations in which the cells of interest constitute only a small percentage of the total population. This method has been used commonly to measure antibody secretion by individual B-cells (26-30) and has also been used with streptococcal protein A to detect antibody-secreting cells (31). With the application of this technique to the study of pituitary cells, several investigators have observed MS cells which were secreting both PRL and GH in the rat anterior pituitary gland (18, 19). In this report, we used the reverse hemolytic plaque assay to examine the effects of chronic estrogen treatment on PRL-secreting cells, GH-secreting cells, and MS cells in rats with transplantable pituitary tumors and in non-tumor-bearing animals. A combination of the reverse hemolytic plaque assay and ICC which confirms the presence of MS cells in normal and neoplastic pituitary tissue is also described.

MATERIALS AND METHODS

Animals. Female 40-day-old Wistar-Furth rats (Harlan Sprague Dawley, Madison, WI) were implanted s.c. in the right flank with a 1-mm³ portion of tumor as described previously (13). The animals were maintained on 12 h light and 12 h darkness and were allowed to feed ad libitum. After 20 to 35 days, a palpable tumor was detected in all animals. The animals were divided into 3 groups and some were treated with 10 mg DES (Sigma Chemical Co., St. Louis, MO) or 10 mg progesterone (Sigma) in Silastic tubings for 3 weeks or received empty Silastic tubings. After 3 weeks, the rats were sacrificed and the tumors were used for study. Non-tumor-bearing 60-day-old female Wistar-Furth rats were implanted with DES capsules for 3, 6, and 9 weeks after which time they were sacrificed and the pituitaries were used for subsequent studies. Control rats with empty Silastic tubings were sacrificed between 80 and 120 days of age. All animals were killed by decapitation between 8 and 10 a.m.

Cell Culture. Anterior pituitaries and tumors were dispersed in 0.25% trypsin in DMEM (Grand Island Biological Company, Grand Island, NY). After tissue was sliced into 1.0-mm fragments, they were rinsed with DMEM to remove the excess RBC and then trypsinized for 45 min at 37°C with gentle stirring. Monodispersed cells were prepared as reported previously (32) and then centrifuged at 250 x g for 5 min. Approximately 1-1.5 x 10⁶ cells/10 mg of pituitary tumor tissue was usually obtained. Viability of pituitary cells and tumor usually exceeded 95%. DMEM with penicillin (10,000 units/ml), streptomycin (10,000 µg/ml), Fungizone (25 µg/ml), 15% horse serum, and 2.5% fetal bovine serum were used to culture the cells for 18-24 h at 37°C in an atmosphere of 5% CO2-95% air.

RHPA. The RHPA procedure was done according to the method of Neil and Frawley et al. (15). oRBC were obtained from Colorado Serum Company (Denver, CO). The oRBC were concentrated by centrifugation at 500 x g for 10 min to give 1.0 ml of packed cells. After 3 washings in saline (0.9 g of NaCl/100 ml of water), 1 ml of protein A (0.1 mg/ml of PBS) (Sigma) and 10 ml of CrCl3 (0.1 mg/ml of saline) were mixed and then added to the 1-ml packed volume of washed oRBC. The cells were incubated for 1 h at 30°C and then washed once with saline and twice with DMEM containing 0.1% BSA (Sigma) and resuspended in 25 ml of DMEM-0.1% BSA for storage at 4°C. Protein A-complexed oRBCs were used within 5 days.

Cultured pituitary cells were removed from the flask by a brief incubation in 0.25% trypsin for 5 min. The assay was conducted in a 30-µl Cunningham incubation chamber (28) constructed on a poly-L-lysine-coated glass microscopic slide. Tape was placed 20 mm apart across the slide and a 22-mm glass coverslip was placed over the two pieces of tape. Equal volumes of monodispersed pituitary cells (1.0 x
10^6/ml) and 5 x 10^6 cell/ml protein A-coated orBC both in DMEM with 0.1% BSA containing 1 mg/ml ascorbic acid were mixed and infused by capillary action into the Cunningham chamber (15, 28). The cells were allowed to attach to the poly-L-lysine-washed slides for 60 min in the CO_2 incubator at 37°C. The unattached cells were then washed from the chamber by placing fresh DMEM-0.1% BSA through one side of the Cunningham chamber and drawing it through to the other side with absorbent paper. The chamber was then filled with a solution of DMEM-0.1% BSA containing rabbit anti-rat PRL or monkey anti-rat GH antiserum at a final dilution of 1:100. Human GRH 1-44 [1 x 10^-7 M; Peninsula Labs (Belmont, CA)] was added to all assays for GH production. The two sources of antiserum used were: (a) a gift from Dr. Frawley, Iowa State University. These antiserum have been well characterized in several publications (18, 20–24); and (b) rabbit anti-rat PRL and monkey anti-rat GH from Dr. A. F. Parlow, distributed through the National Hormone Pituitary Agency. Both hormones from Dr. Frawley were used in the RHPA, while antiserum from both sources were used in the immunocytochemical assay.

After incubation with the antiserum for 2 h at 37°C in a CO_2 incubator, guinea pig serum was added at a final dilution of 1:10 as a source of complement (GIBCO) with 1 mg/ml ascorbic acid in DMEM-0.1% BSA. The slides were returned to the CO_2 incubator for another 60 min after which time the reaction was stopped by filling the chamber with 2% glutaraldehyde-2% formaldehyde in a cacodylate buffer, pH 7.2. After hematoxylin and eosin staining, plaques were identified, quantitated, and photographed with a Zeiss II light microscope.

Combined PRL-GH Assay. One method of estimating the mammomatrotropic cell population included assaying for both PRL and GH in the same population as well as performing separate assays for PRL and GH using the method of Leong et al. (19). In these assays PRL and GH antiserum were both added at a final dilution of 1:100 and human GRH was also included (1 x 10^-7 M). The remainder of the procedure was identical to the previously described one. The differences in the number of PRL and GH cells assayed separately and in combination was assumed to represent the MS cell population.

The specificity of the antiserum was examined by absorbing each antiserum with 100 μg/ml of highly purified NIADDK iodination grade antigen from the National Pituitary Hormone Agency, NIH. Absorption was done by mixing antigen and antibody for 2 h at 23°C followed by 18 h at 4°C then using these in the RHPA. Absorbed antiserum did not produce any plaques in the individual or combined assays. Absorption of PRL antiserum with 100 μg/ml of GH antigen and GH antiserum with 100 μg/ml of PRL did not abolish plaque formation.

Procedure controls for the RHPA included omission of hormone-specific antiserum to guinea pig or monkey or antibody to the hormone of interest. Also, omission of the primary antiserum, the biotinylated IgG, or the avidin-biotin complex peroxidase reagent resulted in no staining. Substitution of rabbit or monkey serum for primary antiserum did not produce staining.

Combined Reverse Hemolytic Plaque Assay and Immunocytochemistry. In some experiments, after the RPHA was completed, the slides were fixed in buffered formalin and then stained for either PRL or GH by ICC. After RHPA with PRL antiserum and GH staining by ICC, the cells which formed plaques and stained for GH by ICC were considered to be MS which produced PRL and contained stored GH by ICC. Likewise, after RHPA with GH antiserum and with PRL staining by ICC, the cells which formed plaques and contained PRL were considered to be MS.

Quantitation and Statistics. A minimum of 200 cells were counted for each slide in the RHPA and scored as positive or negative for plaque formation. For evaluation of the ICC assay, a minimum of 1000 cells/slide was counted by the aid of a 1- x 1-cm^2 grid in the ocular of the microscope. Cells with a dark brown granular precipitate in the cytoplasm were scored as positive in the assay. The difference between the sum of PRL and GH cells in the single RHPA and those in the combined RHPA was considered to represent MS cells. This difference was also analyzed in the ICC assay for PRL and GH cell staining individually and in the combined assay. Finally, the recognition of cells that produced GH and contained immunoreactive PRL and vice versa, analyzed by the sequential RHPA-ICC assays, provided further evidence for the presence of MS cell populations.

Quantitation of plaque areas which was related to the amount of secreted hormone (25) was done with a Bioquant II IBM computer with a digitizing morphometry program and a Zeiss II microscope. A minimum of 100 plaque areas/experiment were examined except in cases in which fewer plaques were available. Statistical differences between groups were evaluated by Student's t test.

Results

DES treatment of non-tumor-bearing rats resulted in an increase in pituitary weight after 3, 6, and 9 weeks as was observed previously (9, 13). After 9 weeks of DES treatment, the pituitaries were 5-6-fold larger than in control rats. Tumor-bearing rats treated with DES for 3 weeks had inhibition of tumor growth compared to control tumor-bearing animals as observed previously (13). Tumors in control rats were usually 20-fold larger than those in DES-treated rats after 3 weeks of treatment.

Hemolytic plaques were easily seen after pituitary cells were incubated with PRL or GH antiserum. Photomicrographs of live cells revealed some plaque-forming cells (Fig. 1). After hematoxylin and eosin staining of fixed cells, the plaque-forming and non-plaque-forming cells (Fig. 2) could be easily quantitated by light microscopy.

The percentage of PRL-secreting cells in non-tumor-bearing rats treated with DES increased after 3 and 6 weeks of DES treatment. There were no significant differences in the percentages of PRL, GH, and MS cells in the pituitaries of non-tumor-bearing rats between 60 and 120 days of age. There was a significant decrease (P < 0.01) in the MS cell population as measured by the RHPA (Fig. 3A). Immunocytochemical staining showed that the percentage of cells and the changes in PRL, GH, and MS cells were almost identical with this technique, which measures stored hormone, as it was with the RHPA technique (Fig. 3B). There was a significant increase in PRL localization of GH and PRL was done by staining with both antiserum concurrently.

The specificity of the antiserum used for ICC was tested by absorption with NIADDK iodination grade antigen. Ten μg/ml of purified GH and PRL abolished ICC staining of their respective antiserum, while PRL or GH staining was not abolished with 100 μg/ml of GH or PRL, respectively. Likewise, omission of the primary antiserum, the biotinylated IgG, or the avidin-biotin complex peroxidase reagent resulted in no staining. Substitution of rabbit or monkey serum for primary antiserum did not produce staining.
cells (P < 0.001) after 6 and 9 weeks of DES treatment as determined by the RHPA and ICC (Fig. 3, A and B). Analysis of plaque areas showed that DES treatment caused a significant increase in the PRL plaque areas after DES treatment for 3, 6, and 9 weeks, while the plaque areas of the GH cells were not significantly different after DES treatment (Fig. 3C).

RHPA analysis of MtT/W15 tumors from untreated rats revealed production of both PRL and GH by the tumor cells. Approximately 12 ± 2.3% (SE) of the tumor cells were MS as judged by the combined assay for PRL and GH (Fig. 4A). After DES treatment for 3 weeks, there was a significant decrease in PRL and a significant increase in GH-producing cells with a decrease in the MS cell population (Fig. 4, A and B). Three weeks of progesterone treatment led to a significant increase in PRL cells, while the GH and MS populations were not significantly different from the untreated MtT/W15 tumors. ICC staining of cytocentrifuged M+T/W15 tumor cells showed more positive PRL cells and less GH-positive cells and MS cells compared to the RHPA analysis (Fig. 4, A and B). Similarly, after DES treatment there was a significant decrease in PRL cells and an increase in GH-positive cells but the MS cells were not significantly different from those in the tumors from untreated rats. Progesterone treatment did not change the PRL, GH, or MS cells as evaluated by ICC. Analysis of plaque areas revealed a significant increase in the areas of GH plaques and a decrease in PRL plaque areas after DES treatment, while progesterone treatment caused a significant increase in PRL plaque areas, but not in the areas of GH cells (Fig. 4C).

The formation of hemolytic plaques was abolished after absorption of the GH and PRL antisera with the appropriate antigens. The numbers of PRL or GH plaques were not changed by absorbing the PRL antibody with GH or PRL, respectively. When the viability of the pituitary cells after the RHPA was evaluated by the ability to exclude trypan blue, more than 95% of pituitary cells excluded the dye as evidence of viability.

The analysis of MS cells was also done by a combination of the RHPA followed by ICC staining. With this technique, it was easy to visualize cells producing PRL or GH which formed hemolytic plaques and also stained for the hormone not used in the RHPA (PRL or GH) after ICC (Fig. 5). The percentage of MS cells was probably underestimated with this method compared to the other methods of estimating MS cells that were used in the study (Table 1). However, this approach confirmed the existence of cells which were secreting one hormone and storing a different hormone thus constituting MS cells. The MS cells decreased significantly after 9 weeks of DES treatment in rats with hyperplastic pituitaries, while the MS cells also decreased significantly with DES treatment in the MtT/W15 tumor-bearing rats when analyzed by the combined RHPA-ICC method.

DISCUSSION

The reverse hemolytic plaque assay which has been used to study antibody production (26-31) has recently been applied to
the study of hormone production by several investigators (15–25). Using this technique, we have shown that chronic estrogen treatment results in an increase in the percentage of PRL-producing cells in the hyperplastic rat pituitary gland, while in the MtT/W15 transplantable tumor there is a marked increase in GH-producing cells and a significant decrease in PRL-producing cells along with the inhibition of tumor growth after 3 weeks of DES treatment.

This study shows that DES has different effects on hormone secretion and storage in hyperplastic pituitaries compared to its effects on established transplantable tumor such as the MtT/W15. Our recent studies on DES-treated MtT/W15 tumors using immunohistochesic staining of tissues (13) and in vitro synthesis of PRL and GH (14) have also shown that DES treatment increases GH cell number and synthesis while decreasing the number of PRL cells and PRL synthesis. In these same studies DES treatment in non-tumor-bearing rats produced an increase in the number of PRL cells and in PRL synthesis. The paradoxical effect of DES in causing an increase in the PRL cells in the animal's own pituitary while producing MS cells these putative transforming factors may be similar to those modulating the corresponding enhancer elements for PRL and GH. Our study has shown that the MtT/W15 tumor contains MS cells, so it is possible that the regulatory factors for GH gene expression become dominant under DES influence while in the normal pituitary the regulatory factors for PRL gene expression is dominant. Tashjian et al. (34) and Boockfor et al. (20) have shown that hydrocortisone also stimulates GH production and decreases PRL production in clonal strains of rat pituitary tumor cells in vitro. Additional studies are needed to understand the mechanisms regulating GH and PRL production in normal and neoplastic pituitary tissues under the influence of estrogens and other steroids.

The effects of estrogen in this study showed a decrease in the MS cells after chronic usage of DES in vivo for 9 weeks, although there was no significant change after 3 and 6 weeks of in vivo DES treatment. A recent report by Boockfor et al. (24) indicated that in vitro treatment with 17β-estradiol for 6 days elevated the percentage of PRL cells that produced hormone by the RHPA. These results are different from our findings for several possible reasons: (a) the in vitro effects of estrogen may be different from the in vivo effects in our experiments; and (b) previous studies by Wiklund et al. (6) have shown that the Holtzman rat, which was used by Boockfor et al. (24), responded poorly even to high doses of DES in vivo.
PRL AND GH PRODUCTION IN PITUITARY TUMORS

![Figure 5](image_url)

Table 1: Analysis of pituitary mammosomatotrophic cells by the reverse hemolytic plaque assay followed by immunocytochemical staining

<table>
<thead>
<tr>
<th>Group</th>
<th>N*</th>
<th>% of mammosomatotrophic cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>6</td>
<td>7.2 ± 1.4*</td>
</tr>
<tr>
<td>DES-3 wk</td>
<td>3</td>
<td>13.0 ± 2.0</td>
</tr>
<tr>
<td>DES-6 wk</td>
<td>6</td>
<td>6.3 ± 1.5</td>
</tr>
<tr>
<td>DES-9 wk</td>
<td>6</td>
<td>2.1 ± 0.63*</td>
</tr>
<tr>
<td>MtT/W15</td>
<td>5</td>
<td>8.4 ± 2.2</td>
</tr>
<tr>
<td>MtT/W15-DES</td>
<td>3</td>
<td>2.0 ± 1.0</td>
</tr>
</tbody>
</table>

* Number of slides analyzed from different experiments. Two slides for each experiment were used. A minimum of 200 cells/slide were analyzed. 
† Mean ± SE. 
| Rats without pituitary tumors were treated with diethylstilbestrol as indicated. |
| Rats with MtT/W15 tumors were treated with diethylstilbestrol for 3 weeks. Inhibition of tumor growth compared to controls was observed during this period. |

with PRL cell proliferation unlike other strains of rats, such as F344 and Wistar-Furth. Another possible explanation for the decrease in MS cells after chronic DES treatment might include interconversion of cell types as suggested by Strautman et al. (35) who reported on the conversion of somatotrophs to mammosomatotrophic cells. Several lines of evidence have suggested that the MS cell in normal pituitary is a transitional cell type which is capable of producing GH and PRL (35, 40). Recent ultrastructural immunohistochemical studies have revealed various subtypes of PRL cells in the rat anterior pituitary gland (41) as well as MS cells (42). MS cells have also been described in other species (43) and have also been localized in human pituitary tumors by several investigators (44, 45). Recent studies by Fumagalli and Zanini (43) in the bovine pituitary gland revealed both GH and PRL in MS cells as separate granules as well as in the same secretory granules suggesting that in these cells the two hormones were processed in the same Golgi cisternae and that mechanisms exist to separate the two hormones from each other.

The RHPA adds another dimension to the study of normal and neoplastic pituitary cells, since it measures hormone release by individual cells. Because of the possible differences between hormone storage and release due to the variable molecular forms of GH and PRL and other unknown factors (46, 47), it is possible that the storage forms of these hormones may be different from the secreted forms. Thus, a combination of techniques, such as the RHPA, immunocytochemistry, and ultrastructural studies, will contribute to a better understanding of the diversity of PRL and GH storage and secretion.

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