IMMUNOHISTOCHEMICAL LOCALIZATION OF FSH AND LH IN THE HUMAN PITUITARY GLANDS

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The localization of follicle stimulating hormone (FSH) and luteinizing hormone (LH) in human pituitary gland was studied using the double staining technique of the indirect immunoenzyme method. Human pituitary specimens obtained from 7 adult patients were fixed with 10% buffered formalin solution. Anti-swine FSH (sFSH) and anti-human chorionic gonadotropin-β subunit (hCGβ) antiserum were used for the present investigation. 3,3'-diaminobenzidine (DAB), 4-Chloro-1-naphthol and 4-Chloro-1-naphthol-pyronine were employed for the substrates of horseradish peroxidase (HRP). FSH containing cells appeared as oval and polygonal type cells, while LH containing cells were almost all oval type, smaller, and fewer in number than FSH containing cells. After decolorization with ethanol and staining with resorcin, fuchsin and azan, FSH and LH containing cells appeared to be Romeis γ-cell and δ-cell, respectively.

There has been much controversy over whether FSH and LH appear in the same or different cells of the anterior pituitary gland. Studies in rat and human pituitary using immunohistochemical staining reported that FSH and LH were contained in the same cells (1, 4, 6, 8). Recently, Purandare et al. (9) demonstrated two gonadotropic cell types (FSH and LH) in rat pituitary gland by employing anti-FSH-β subunit and anti-LH-β subunit antiserum, using the immunoenzyme technique.

Significantly, abnormal serum levels of FSH and/or LH are often experienced in the clinical course of patients with testicular diseases. Therefore, clarifying the localization of FSH and LH in the pituitary gland would help our understanding of the secretion mechanism of the pituitary gonadotropin. Hence, the aim of the present experiment is to delineate FSH containing cells and LH containing cells in the human pituitary glands, using the double staining technique of the immunoenzyme method.

MATERIALS AND METHODS

Preparation of Specimens: Human pituitary glands were obtained within 4 hr post-mortem from autopsies of 7 adults without hormonal diseases. All of the pituitary preparations were fixed in 10% buffered formalin for 24 hr, dehydrated and embedded in paraffin.
Antisera: Antiserum against swine FSH (sFSH, Calbiochem Lot. No 500218) was prepared by immunizing white male rabbits (seven times at 2 week intervals) intradermally in the foot pad or the back with 2 ml emulsion containing 1 ml complete Freund’s adjuvant (Difco) and sFSH (1 mg/ml). Titration of the anti-sFSH antiserum was performed by the immuno-double diffusion method. The rabbit anti-sFSH antiserum produced a broad precipitin line against sFSH (1 mg/ml) up to eight-fold dilution, and no precipitin line was seen against hCG by double diffusion technique. Then the anti-sFSH antiserum was adsorbed with hCG, normal swine serum (Flow-Laboratories) and human testicular powder extracted with acetone. By means of immunoelectrophoresis, anti-sFSH antiserum alone showed a broad and dim precipitin line against sFSH (1 mg/ml), but anti-sFSH antiserum adsorbed with hCG, normal swine serum and human testicular powder produced only a thin precipitin line (Fig. 1).

Anti-hCGβ antiserum (Mochida) previously adsorbed with sFSH was used against human LH.

The specificities of anti-sFSH and hCGβ antisera were experimented by staining human anterior pituitary glands. For control studies, anti-sFSH and hCGβ antiserum previously absorbed with human menopausal gonadotropin (hMG, N.V. Organone) and hCG, respectively, were applied to the same preparation. All of the mixtures of antisera and antigens were incubated at 37°C for 1 hr, stored at 4°C for 5 days and then centrifuged.

**Horseradish peroxidase labeled goat anti-rabbit y-globulin antibodies (HRP-Conjugate):** Preparation of the HRP-conjugate was made according to the method of Nakane and Kawaoi (7).

**FSH and LH containing cells in the human pituitary glands:** FSH containing cells and LH containing cells were identified using the double staining technique of the indirect immunoenzyme method. The double staining was performed.

Fig. 1. Immunoelectrophoretic patterns of anti-sFSH antiserum. A broad and dim precipitin line was observed by anti-sFSH antiserum. After adsorption with hCG, normal swine serum and human testicular powder only a faint precipitin line was produced (arrows).
in the following manner: Thin sections of human pituitary were incubated with rabbit anti-sFSH or hCGβ antiserum at room temperature for 30 min, then free immunoglobulin was washed out by 0.001 M phosphate buffered saline (PBS), pH 7.2. They were incubated with HRP-conjugate at room temperature for 30 min. HRP was reacted by DAB or 4-Cl-1-naphthol, followed by pyronin staining of the products (4-Cl-1-naphthol-pyronin). Afterward immunocomplex of rabbit immunoglobulin and HRP-conjugate was dissociated from pituitary sections using 2 M glycine-HCl buffer, pH 2.0 at room temperature for 36 hr. The dissociated and washed sections were again reacted with more anti-hormone antiserum and HRP-conjugate. For a solution of the substrates, 60 mg naphthol was dissolved in 3 ml pure ethanol, then mixed with PBS, pH 7.2 containing 0.005% H₂O₂. The reaction products of DAB and 4-Cl-1-naphthol appeared brown and blue, respectively. Following blue staining of 4-Cl-1-naphthol, the same tissue was stained pink by pyronin. The human pituitary sections of blue and pink staining were decolorized with pure ethanol after dissociation of immunocomplex. Afterward these sections were stained with resorcin, fuchsin and azan.

RESULTS

The FSH containing cells are shown in Fig. 2a. Positive products were remarkably weak when reacted with anti-sFSH antiserum previously adsorbed with hMG (Fig. 2b).

The LH containing cells strongly reacted with anti-hCGβ antiserum which was previously adsorbed with sFSH (Fig. 3b). When anti-hCGβ antiserum was

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**FIG. 2a.** Positive products with anti-sFSH antiserum are shown in many polygonal cells in human pituitary gland. ×210

**FIG. 2b.** Serial section to Fig. 2a. No positive cells were immunostained with anti-sFSH antiserum after adsorption with hMG. ×210
FIG. 3a. Positive products with anti-hCGβ antiserum were shown in many oval cells in human pituitary gland. ×210

FIG. 3b. Serial section to Fig. 3a. No positive cells were immunostained with anti-hCGβ antiserum after adsorption with hCG. ×210

FIG. 4. A section of peripheral area of human pituitary gland immunohistochemically stained for FSH and LH. Cells reacted with anti-sFSH antiserum stained pink and cells reacted with anti-hCGβ antiserum stained blue. The FSH containing cells are large polygonal type and more numerous than the (small and oval) LH containing cells. ×420
adsorbed with hCG, no positive reaction products were observed (Fig. 3b).

In general, large numbers of both FSH and LH containing cells were situated at the pars distalis and peripheral area of the adult human pituitary glands, while at the central area they tended to decrease. The larger number of FSH containing cells were usually a large polygonal type whereas LH containing cells were small and oval (Fig. 4). Cells reacted with anti-sFSH antiserum were stained blue and

Fig. 5a, b. The same area of same section of a human pituitary. Immunohistochemically the FSH containing cells are stained blue and LH containing cells pink in Fig. 5a. The FSH containing cells stained with resorcine, fuchsin and azan show light purple and the LH containing cells aniline blue in Fig. 5b. ×420
cells reacted with anti-hCGβ antiserum were stained pink (Fig. 5a). When the same section was stained with resorcin, fuchsin and azan, the FSH containing cells stained light purple and the LH containing cells stained aniline blue (Fig. 5b).

**DISCUSSION**

In a study by Salamonsen *et al.* (10), using radioimmunoassay anti-human FSH antiserum cross-reacted with swine FSH. Our anti-sFSH antiserum was used after adsorption with hCG, normal swine serum and human testicular powder. When this antiserum was reacted with sFSH (1 mg/ml) immunoelectrophoretically, only one precipitin line was observed. After adsorption of this antiserum with hMG, the positive reaction products in the human pituitary markedly decreased. Therefore this anti-sFSH antiserum was considered to have a specific reactivity against human FSH.

It has been demonstrated that β subunit of hCG and human LH are very similar and unlike those of other glycoprotein hormone (3, 5) and anti-hCG antiserum cross-reacts with human LH (11, 12). In this experiment, anti-hCGβ antiserum adsorbed with sFSH was used.

In order to obtain two clear colors, 60 mg 4-Cl-1-naphthol was dissolved in 3 ml pure ethanol and then mixed with 100 ml PBS at pH 7.2 containing 0.005% H₂O₂ at 24 hr before use. Pyronine staining was applied to the products of 4-Cl-1-naphthol, because no products of α-naphthol could be observed in the human pituitary sections. The pink of 4-Cl-1-naphtholpyronine was stronger than that of α-naphthol.

Using our antisera and the substrate solutions for HRP, the FSH containing cells and the LH containing cells were clearly distinguished in the human pituitary glands. Our observations differed from other reports that FSH and LH appeared to occur in the same cells of the pituitary gland (1, 4, 6, 8). Phifer (8) showed by the immunoglobulin-peroxidase bridge method using specific FSH and LH antibodies that the gonadotropic cells situated at the pars distalis of the human pituitary glands contained both FSH and LH. Reasons why the two colors overlap at the same cell and/or site in the double staining of the indirect immunoenzyme method could be: 1) both antisera are cross-reactive; 2) the antisera are contaminated with other antibodies; 3) immunocomplex of the first staining is incompletely dissociated; 4) overreaction of enzyme activity; 5) inadequate fixation of the antigen followed by its diffusion; and 7) both antigens are localized at the same cell and site. Therefore, when the two colors overlap on the same cell and site, it is rather difficult to conclude the simultaneous localization of different antigens. However in our experiment the FSH containing cells and LH containing cells clearly showed different colors. The cell types of FSH and LH containing cells immunostained with anti-sFSH and hCGβ antiserum agreed with the findings of Purauder *et al.* (9), though the numbers of FSH and LH containing cells differed from their observation. In our study the numbers of FSH containing cells were larger than those of LH containing cells.

Leleux and Robyn (2) previously described that by resorcin, fuchsin and Heidenhains azan staining, the pituitary cells were separated into 5 types—α (red), ε (orange), β (browne violet), δ (aniline blue) and γ (light purple)—and there is experimental evidence supporting the localization of adrenocorticotropic hormone.
in the basophils of Romeis $\beta$ type, somatotrophic hormone in the acidophils of Romeis $\alpha$ type, prolactin in the acidophils of Romeis $\varepsilon$ type and gonadotropic hormone (FSH and LH) in the basophils of Romeis $\delta$ type. We attempted to compare the immunohistochemistry with resorcin, fuchsin and azan staining in the same section. The results showed that the FSH containing cells and the LH containing cells corresponded with Romeis $\gamma$-cell and $\delta$-cell, respectively.

REFERENCES