IMMUNOCYTOCHEMICAL LOCALIZATION OF PROGESTERONE IN THE MOUSE ADRENOCORTICAL ADENOMA CELLS (Y-1)*

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Indirect immunofluorescence and immunoperoxidase techniques were successful in demonstration of progesterone in the cultured mouse adrenocortical adenoma cells (Y-1). The immunoreactive progesterone was localized in the cytoplasm, but not in the nuclei, and showed increased staining in the cells stimulated by either adrenocorticotrophic hormone (ACTH) or dibutyryl adenosine 3', 5'-cyclic monophosphate (dbc AMP). "Round up" cells, which are considered to be the most active form of steroidogenesis, revealed intensive staining.

The mode of localization in the cytoplasm of the immunoreactive steroid will be discussed.

Recent successes in the immunohistochemical and cytochemical localizations of the peptide hormones, such as the hypothalamic releasing factors (12), anterior pituitary hormones (10), pancreatic islet hormones (3), and gastro-intestinal polypeptides (5), have led to rapid progress in the functional morphology of endocrine cells and organs. On the contrary however, the demonstration of steroid hormones by this method meets with technical difficulties and few reports have hitherto been presented (1, 4, 6).

Reported herein are the results of immunocytochemical observation of the cultured mouse adrenocortical adenoma cells (Y-1) (2), where progesterone, the main product of the cells, was successfully localized in the cytoplasm of these cells using specific antisera to the hormone.

MATERIALS AND METHODS

The mouse adrenocortical adenoma cell line, clone Y-1 (2) was purchased from ATCC through Dainippon Pharmaceutical Co. Ltd., Tokyo, which was grown in Ham F-10 (Flow Lab. Inc.) supplemented with 10% fetal calf serum (Gibco) in either 35 mm diameter sterile plastic Petri dishes (Lux Scientific Co.) or the culture bottles with glass cover slips, 40 × 10 mm (Matsunami Glass Industries, Ltd., Osaka), at 37°C in a water-saturated atmosphere of 5% CO₂ in air. The Y-1 cells were stimulated by cultivating them in the medium containing either 1 mU/ml ACTH

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(Armour) or 1 mM dibutyryl adenosine 3', 5'-cyclic monophosphate (dbc AMP, Boehringer). The amount of corticosteroids (CS) secreted in the medium was measured by fluorometry (14) and in part by radioimmunoassay. The time course response of steroidogenesis is shown in Fig. 1 (7). The result of radioimmunoassay for progesterone is demonstrated in the Fig. 2.

The anti-progesterone rabbit antisera and the anti-cortisol rabbit antisera were kindly offered by Dr. A. Kanbegawa, Teikoku Hormone MFG. Co. Ltd., Tokyo. These antisera were incubated with 10 mg/ml bovine serum albumin (BSA) at 37°C for 60 min and thereafter at 4°C overnight, in order to extinguish their anti-BSA activity. The rabbit anti-BSA antisera were the gift of Dr. A. Ishibashi, Department of Microbiology, Nihon University School of Medicine, Tokyo. The anti-rabbit IgG goat γ -globulin labeled with fluorescein isothiocyanate (FITC) (9, 13) or with horseradish peroxidase (11) were prepared in this laboratory.

Immune stainings.

The immunofluorescence method (IF) was applied to the Y-1 cells grown on glass cover-slips in culture bottles. For immunoperoxidase staining (IP) the Y-1 cells monolayered in the plastic Petri dishes were used. The cells were gently washed with 0.01 M phosphate buffered saline, pH 7.2 (PBS), and immersed in

FIG. 1. The time course response of steroidogenesis in the Y-1 cells unstimulated (●---●) or stimulated by either ACTH, 1 mU/ml (●—●) or dbc AMP, 1 mM (○—○) (7).

FIG. 2. The amount of progesterone (ng/ml) assayed by RIA in the medium without the Y-1 cells (Medium), with the Y-1 cells cultured in the stimulant free medium (Control), stimulated by ACTH, 1 mU/ml, for 24 hr (ACTH), or by dbc AMP, 1 mM, for 24 hr (dbc AMP).

various kinds of fixatives (Table 1) for 30 min at 4°C. After washing with cold PBS the cells were incubated with one of the following sera for 60 min at room temperature or at 37°C: (a) Anti-progesterone antisera absorbed with BSA and diluted to 1 : 20 in 1% BSA. (b) Anti-progesterone antisera absorbed with BSA and progesterone (10 µg/ml) and diluted to 1 : 20 in 1% BSA. (c) Anti-cortisol antisera absorbed with BSA and diluted to 1 : 20 in 1% BSA. (d) Anti-BSA antisera diluted to 1 : 10 in PBS. (e) Nonimmunized rabbit sera diluted to 1 : 10 in 1% BSA. The incubation was ended by immersion in cold PBS followed by the second reaction with either FITC labeled anti-rabbit IgG goat γ-globulin for 60 min at 37°C or peroxidase labeled anti-rabbit IgG goat γ-globulin for 30 min at room temperature. After washing with cold PBS the specimens stained by IF were observed with a fluorescence microscope (Zeiss Illuminator I equipped with the excitor filter UG 1 and the barrier filter 44). The specimens for IP were

colored by further incubation in the Karnovsky's solution for 10–20 min at room temperature, and observed under conventional light microscopy.

RESULTS

The effects of fixatives on the immune staining of anti-progesterone antisera (a) are demonstrated in the Table 1. The 2.5 to 10% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, and 2% paraformaldehyde in phosphate buffer with picric acid [Zamboni's solution (15)] resulted in relatively reproducible stainings. On the contrary, the cells which were fixed by 95% ethanol yielded no positive results. Therefore, in the present study, Zamboni's solution was employed as a standard fixative, with its stable effect on the specific staining and less marked nonspecific reaction.

Both IF and IP gave essentially the same staining results: The Y-1 cells unstimulated or stimulated by either ACTH or dbc AMP were positively stained by the antisera (a) with a variable intensity (Figs. 3, 5, 7, 9, 10, 11 and 12). Their stainings were significantly decreased when the antisera (a) were replaced by the antisera (b) (Fig. 8). The cells reacted with the sera (c), (d) and (e) showed no positive staining whether they were stimulated or not (Figs. 4 and 6).

The intensity of the staining of the individual cells and the population of positive cells correlated well with the amount of CS secreted in the media as measured by fluorometry or RIA. The unstimulated cells showed flat and polygonal shapes, and only a few were weakly stained (Fig. 3). In contrast, a majority of the cells which were stimulated by either ACTH or dbc AMP and took the shape of "rounding up" revealed intensive staining of their cytoplasm, indicating the presence of abundant immunoreactive progesterone (Figs. 5, 7, 9 and 10). It was noteworthy however, that some of the stimulated cells which were still polygonal in shape clearly demonstrated positive staining (Fig. 5).

There were two patterns of cytoplasmic staining in the rounded cells; one with homogeneous perinuclear localization (Fig. 9) and another with peripheral

TABLE 1. *Effects of fixatives on the immune staining of progesterone in the Y-1 cells*

Fixatives	(%)	Staining
Ethanol	95	—
Glutaraldehyde	1	— to ±
Glutaraldehyde	2.5	+
Glutaraldehyde	5	+ to ++
Glutaraldehyde	10	+
Glutaraldehyde	20	±
Formaldehyde	1	—
Formaldehyde	5	±
Formaldehyde	10	±
Paraformaldehyde (15)	2	+
None		—

FIG. 3. The Y-1 cells in unstimulated state. Some of the polygonal cells are weakly stained for progesterone (IP). $\times 300$

FIG. 4. The Y-1 cells stimulated with ACTH (1 mU/ml) for 24 hr. No positive staining appeared by incubation with nonimmune rabbit serum (IP). $\times 300$

FIG. 5. The Y-1 cells stimulated with ACTH (1 mU/ml) for 24 hr. Some, especially those rounded, show a positive staining for progesterone (IP). $\times 300$.

FIG. 6. The Y-1 cells stimulated with ACTH (1 mU/ml) for 24 hr. Incubation with anti-cortisol gives no positive staining (IP). $\times 300$

FIG. 7. The Y-1 cells stimulated with dbc AMP (1 mM) for 4 hr and 30 min. There is a mixture of anti-progesterone reactive and non-reactive cells (IP). $\times 300$

FIG. 8. The Y-1 cells stimulated with dbc AMP (1 mM) for 24 hr reacted with anti-progesterone antiserum previously absorbed by progesterone (IP). The intensity of the staining is markedly decreased as compared with those in the Fig. 7. $\times 300$

FIG. 9. The Y-1 cells stimulated with dbc AMP (1 mM) for 24 hr. The perinuclear cytoplasm shows a heavy reaction with anti-progesterone (IP). $\times 600$

FIG. 10. The Y-1 cells stimulated with ACTH (1 mU/ml) for 24 hr. Note the peripheral and granular staining for progesterone (IP). $\times 600$

FIG. 11. The Y-1 cells unstimulated. A few of the polygonal or spindric cells show positive staining for progesterone (IF). $\times 380$

FIG. 12. The Y-1 cells stimulated with dbc AMP (1 mM) for 24 hr. Two rounded cells show strong fluorescence for progesterone (IF). $\times 380$

and coarse granular or rugged reaction (Fig. 10). No positive staining was observed in the nuclei of the cells.

The IF was disadvantageous in that the cells, especially those rounded, were easily lost from the glass cover-slips during the staining processes (Fig. 12), and in that the identification of the negative cells was rather difficult with their minimum background staining (Fig. 11).

DISCUSSION

In spite of the fact that a number of antisera against steroid hormones which have high quality specificity and titer are available at present, there is still a paucity of reports on the immunohistochemical demonstration of these hormones (1, 4, 6). Indeed, there are no fixatives which are able to fix with certainty the steroids themselves in the cells or tissues for the immunochemical reaction. However, if the enzymes which locate in the microsomal or mitochondrial fraction and catalyze the biosynthesis of the steroids are properly fixed, the steroid hormones or their intermediate forms bound to these enzymes could be indirectly stabilized in the cells.

Cultured mouse adrenocortical adenoma cells Y-1 were used for the immunocytochemical demonstration of progesterone because they maintain the ability to secrete corticosteroids of mainly progesterone in response to ACTH or dbc AMP (8), and because their functional states are easily followed by both morphological observation and by measuring CS secreted in the medium (7, 8, 16, 17). With the above-mentioned concept as a basis, several fixatives were applied to the Y-1 cells, among which 2.5 to 10% glutaraldehyde and 2% paraformaldehyde solutions showed a positive and reproducible result in immune staining for progesterone.

The specificity of the immune staining in the present study was supported by the following facts: 1. Progesterone, which is the main product of the Y-1 cells, was positively stained by the specific antisera qualified in RIA system. 2. The intensity of the staining significantly decreased when the antisera absorbed with progesterone was used. 3. Cortisol, which is a minimum component of steroid hormones produced by the murine adrenal cortex, was not stained by anti-cortisol antisera. 4. Both anti-BSA antisera and nonimmune rabbit sera failed to show any positive staining. 5. The intensity and number of the positive cells correlated well with the amount of CS secreted in the media, and the rounded cells which are thought to be the most active form in steroidogenesis were heavily stained. In addition, it is evident that progesterone localized by the immune staining in the present study was synthesized *in situ* and not absorbed from the medium, since the medium itself contained only a trace amount of it (Fig. 2).

The intracytoplasmic localization of the immunoreactive steroid was in accordance with the results of previous reports (1, 4, 6). Bubenik et al. (1) reported the highest concentration of androgen in the interstitial tissue of the testes of both rat and squirrel monkey and a significant amount of it in the seminiferous tubules. They suggested the possibilities of either *de novo* synthesis of the immunoreactive androgen in the tubules or active uptake of the steroids intermediate in the biosynthesis of testosterone.

Goodfriend et al. (4) demonstrated the localization of estrone in the cytoplasm

of the perifollicular cells of rat ovary stimulated by FSH. However, they lacked in use of control stainings, including the absorption test.

The authors (6) attempted to localize deoxycorticosterone (DOC) by IF in the rat adrenal cortex which was fixed with 2.5 to 5% glutaraldehyde. The IF positive cells were distributed throughout the cortex except for the subcapsular layer and showed homogeneous cytoplasmic fluorescence.

There is no morphological evidence for the mode of the intracytoplasmic localization of progesterone in the Y-1 cells. According to Kowal et al. (8), progesterone is synthesized in the Y-1 cells by pregnenolone where Δ^5 - 3β -OH-dehydrogenase and Δ^5 - Δ^4 -isomerase in the microsome are involved. Furthermore, under the actions of 20α -dehydrogenase in microsome and 11β -hydroxylase in mitochondria, 20α -dihydroprogesterone and 11β -hydroxyprogesterone are formed. Therefore, it is likely that the immunoreactive progesterone demonstrated in the present study might be located in the microsomal or mitochondrial fraction binding with these enzymes. Evidence for the detailed subcellular localization of steroid hormones will emerge by immunoelectron microscopical observation.

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