Light and Electron Microscopic Immunohistochemistry on the Localization of Cytochrome P-450 of the Side Chain Cleavage System and of Cytochrome P-450 of 11β-Hydroxylase in the Bovine Adrenal Cortical Cells*

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Summary. In order to know the mechanism of steroid hormone biosynthesis, the localization of cytochromes P-450 of cholesterol side-chain cleavage enzymes (P-450_{SCC}) and P-450 of 11 β-hydroxylase (P-450_{11β}) were studied in bovine adrenal glands by light as well as electron microscopic immunohistochemistry, using monoclonal antibodies. With light microscopy the cytoplasm of the glomerulosa cells was faintly immunostained, while that of the fasciculata-reticularis cells was intensely immunostained by both monoclonal antibodies for cytochrome P-450_{SCC} and cytochrome P-450_{11β}, though the capsular connective tissue cells and the adrenal medullary cells were entirely negative for these reactions. Electron microscopic immunohistochemistry revealed that the positive reaction products for cytochromes P-450 were present on the matrix side of the inner mitochondrial membrane including the tubulovesicular cristae of the cortical cells, and especially of the fasciculata and reticularis cells. The present results indicate that both cholesterol side-chain cleaving enzymes and 11 β-hydroxylase are present in the inner mitochondrial membrane of bovine adrenal cortical cells.

Mechanisms of the biosynthesis of steroid hormones in steroid-secretory cells such as adrenal cortical cells have so far been explained mainly on the basis of data obtained by biochemical methods (MCKERNS, 1969; HEFTMANN, 1970). One of the reasons why morphological methods have made only poor contributions toward elucidating the mechanism of steroid biosynthesis is that the electron microscopic autoradiography is not suitable for this purpose, as steroids are easily lost in the course of tissue preparation. Immunohistochemistry, which is a powerful method for the detection of peptide materials, has also failed to bring any definitive results with the steroidogenesis, mainly because of the difficulties in the fixation of steroids and obtaining the specific antibodies. In order to overcome these limitations, another immunohistochemical approach using antibodies against enzyme proteins engaged in steroid biosynthesis was considered. Actually, only one paper based on this hypothesis has been published (MITANI et al., 1982).

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The present study deals with light as well as electron microscopic immunohistochemistry for cytochromes P-450 of cholesterol side-chain cleaving enzymes and of 11\(\beta\)-hydroxylase in the bovine adrenal cortex, using their monoclonal antibodies.

MATERIALS AND METHODS

Fresh bovine adrenal glands obtained from a slaughterhouse were cut into small pieces and fixed in 4% paraformaldehyde buffered at pH 7.4 with Millonig's phosphate for 12 hrs at 4°C. For light microscopic immunohistochemistry, fixed tissues were washed in phosphate-buffered-saline (PBS) containing 10–20% sucrose for 8 hrs at 4°C. Frozen sections of a 6 \(\mu\)m thickness, mounted on glass slides, were treated with monoclonal antibodies (diluted at 1:100–1:1,000) for 36–72 hrs at 4°C, then rinsed in PBS and finally incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Miles-Yeda) for 12 hrs at 4°C. After rinsing in PBS, the sections were treated with a medium containing 0.005% 3,3' -diaminobenzidine (DAB) and 0.03% \(\text{H}_2\text{O}_2\) for 5–10 min at room temperature. The sections were examined and photographed with a Zeiss Photomicroscope III. For electron microscopic immunohistochemistry, the fixed tissue samples were embedded in 10% gelatin dissolved in PBS and frozen sections were made. The sections were rinsed and dipped in a solution of antibodies as described above. After

![Fig. 1.](image1.png)  
**Fig. 1.** Electron micrograph of mitochondria in a zona glomerulosa cell of the bovine adrenal cortex. Most mitochondria are elongated in shape and have tubular cristae. \(\times 36,000\)

![Fig. 2.](image2.png)  
**Fig. 2.** Electron micrograph of mitochondria in a zona fasciculata cell of the bovine adrenal cortex. Most mitochondria are round or oval in shape and have tubulovesicular cristae. \(\times 30,000\)
incubation with DAB solution, the sections were fixed in 1% OsO₄ solution, dehydrated with a graded series of ethanol concentrations and embedded in Epon epoxy resin. Ultrathin sections cut on a Porter-Blum ultramicrotome were examined in a Hitachi H-500 electron microscope without staining. Control sections were treated with pre-immune mouse serum instead of the monoclonal antibodies. The purification of cytochromes P-450 of cholesterol side-chain cleaving enzymes (cytochrome P-450scc), and of 11β-hydroxylase (cytochrome P-450₁₁β), and the production, purification and specificity of the monoclonal antibodies have been previously described (SUGANO et al., 1985).

For conventional electron microscopy, small pieces of the fresh adrenal cortex were fixed in 2.5% glutaraldehyde solution buffered at pH 7.4 with Millonig’s phosphate for 1 hr at 4°C and postfixed in 1% OsO₄ buffered at pH 7.4 with Millonig’s phosphate for 1 hr at 4°C. After dehydration in graded concentrations of ethanol, the tissues were embedded in Epon epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined in a Hitachi H-500 electron microscope.

Fig. 3. a and b. Light microscopic immunohistochemistry of cytochrome P-450scc in the bovine adrenal cortex. Cells of the zona glomerulosa are faintly immunostained but those of the zona fasciculata (F) and reticularis (R) are intensely stained. The connective tissue cells of the capsule (C) and the adrenal medullary cells (M) are entirely negative. ×60

Fig. 4. a and b. Light microscopic immunohistochemistry of cytochrome P-450₁₁β in the bovine adrenal cortex. The pattern of the immunostaining is same as the situation for P-450scc. G zona glomerulosa, F zona fasciculata, R zona reticularis, C capsule, M adrenal medulla. ×60
RESULTS

Conventional electron microscopy
Bovine adrenal cortical cells have numerous mitochondria, whose number per cell is almost the same throughout the three cortical zones. These cyto-organelles in the zona-glomerulosa cells are mostly elongated in shape (Fig. 1). Their cristae are usually tubular but sometimes lamellar. In all the cells of the zona fasciculata and reticularis, mitochondria are round or oval in shape and have tubular or tubulo-vesicular cristae (Fig. 2).

Immunohistochemistry
In light microscopy, the fasciculata as well as the reticularis cells were intensely immunostained by both monoclonal antibodies for cytochrome P-450$_{SCC}$ and cytochrome P-450$_{11\beta}$ but the glomerulosa cells were only faintly stained by them (Fig. 3, 4). The connective tissue cells of the capsule and the adrenal medullary cells were entirely negative for this immunostaining. At the electron microscopic level, positive reaction

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**Fig. 5.** Electron microscopic immunohistochemistry of cytochrome P-450$_{SCC}$ of the adrenal cortical cells. Reaction products for the enzyme are localized on the matrix side of the inner membranes including the tubulovesicular cristae of some mitochondria (arrow). $\times 28,000$

**Fig. 6.** Electron microscopic immunohistochemistry of cytochrome P-450$_{11\beta}$ of the adrenal cortical cells. Reaction products are localized on the matrix side of the inner membranes including tubulovesicular cristae of some mitochondria (arrow). $\times 39,000$
products for cytochrome P-450scc and cytochrome P-450_{11β} were exclusively localized on the matrix side of the inner membranes of the mitochondria including the tubulovesicular cristae in all the cortical cells, especially in the fasciculata and reticularis cells (Fig. 5, 6). Though each cortical cell had both positively and negatively stained mitochondria, no prominent ultrastructural differences were recognized between the two populations of mitochondria.

All other organelles such as tubular elements of smooth endoplasmic reticulum, lipid droplets and the Golgi apparatus were entirely negative for the immunostaining. In control sections, the immunoreaction was completely negative on all the cytoorganelles, including the mitochondria.

**DISCUSSION**

The present results obtained by electron microscopic immunohistochemistry using monoclonal antibodies are compatible with the biochemical data that cholesterol side-chain cleaving enzymes and 11β-hydroxylase are located in mitochondrial inner membranes including cristae (SATRE et al., 1969; YAGO and Ichii, 1969), and confirm the immunohistochemical results using polyclonal antibodies by Mitani et al. (1982). Namely, the present results indicate that the site of conversion from cholesterol to pregnenolone, from 11-deoxycortisol to cortisol, and from 11-deoxycorticosterone to corticosterone is the mitochondrial inner membrane, because cytochromes P-450_{scc} and P-450_{11β} are substrate-specific parts of the enzyme complexes (SHIKITA and HALL, 1973; TAKEMORI et al., 1975).

As to the coexistence of two populations of mitochondria that are immunostained either positively or negatively, Mitani et al. (1982) proposed that the different immunoreactivity reflects the functional heterogeneity for steroidogenesis. However, this seems unlikely, because no structural differences in the mitochondrial cristae, considered to reflect the functional state of mitochondria for steroidogenesis (Fujita, 1974), were seen between positively-stained mitochondria and negatively-stained ones. Other factors such as the permeability of the antibodies through the plasma as well as mitochondrial membranes or the conditions of tissue fixation may alter the results of immunostaining, although it must be said that the intensity of the immunostaining depends on the content of the enzymes present in the mitochondrial membranes.

The results of light microscopic immunohistochemistry, in which the glomerulosa cells were faintly stained while both fasciculata and reticularis cells were intensely stained, may arise from the biochemical fact that the total content of cytochromes P-450_{scc} and P-450_{11β} in the mitochondria of the fasciculata cells is about twice that of the glomerulosa cells (ICHIKAWA et al., 1970; YAGI et al., 1983). Structural differences of the cristae between the mitochondria of the glomerulosa cells and those of the fasciculata-reticularis cells may also be related to the content of the enzymes.

At any rate, immunohistochemistry for the localization of enzyme proteins is considered to be an excellent method for morphologically detecting the mechanism of steroid biosynthesis.
REFERENCES

Fujita, H.: Adrenal cortex. In: (ed. by) K. Kurosumi and H. Fujita: Functional morphology of endocrine glands. Igaku-Shoin, Tokyo, 1974 (p. 299–342).

Heftmann, E.: Steroid biochemistry. Academic Press, New York. 1970.

Ichikawa, Y., M. Kuroda and T. Yamano: Zonation of hemoprotein P-450 and cytochrome b\(_5\) in the adrenocortex of mammals. J. Cell Biol. 45: 640–643 (1970).

McKerns, K. W.: Steroid hormones and metabolism. Appleton-Century-Crofts, New York, 1969.

Mitani, F., T. Shimizu, R. Ueno, Y. Ishimura, S. Izumi, N. Komatsu and K. Watanaba: Cytochrome P-450\(_{11\beta}\) and P-450\(_{SCC}\) in adrenal cortex: Zonal distribution and intramitochondrial localization by the horseradish peroxidase-labeled antibody method. J. Histochem. Cytochem. 30: 1066–1074 (1982).

Satre, M., P. V. Vignais and S. Idelman: Distribution of the steroid 11-\(\beta\)-hydroxylase and the cytochrome P-450 in membranes of beef adrenal cortex mitochondria. FEBS Lett. 5: 135–140 (1969).

Shikita, M. and P. F. Hall: Cytochrome P-450 from bovine adrenocortical mitochondria: an enzyme for the side chain cleavage of cholesterol. II. Subunit structure. J. biol. Chem. 248: 5605–5609 (1973).

Sugano, S., T. Onishi, N. Hatae, K. Ishimura, H. Fujita, T. Yamano and M. Okamoto: Monoclonal antibodies against bovine adrenal cytochrome P-450\(_{11\beta}\) and cytochrome P-450\(_{SCC}\). Their isolation, characterization and application to immunohistochemical analysis of adrenal cortex. J. Steroid Biochem. (1985, in press).

Takemori, S., H. Sato, T. Gomi, K. Suhara and M. Katagiri: Purification and properties of cytochrome P-450\(_{11\beta}\) from adrenocortical mitochondria. Biochem. biophys. Res. Commun. 67: 1151–1157 (1975).

Yagi, J., T. Sugiyama, M. Okamoto, K. Kurachi and T. Yamano: Zonal distribution of cytochrome P-450s (P-450\(_{11\beta}\) and P-450\(_{SCC}\)) and their relation to steroidogenesis in bovine adrenal cortex. J. Steroid Biochem. 18: 707–713 (1983).

Yago, N. and S. Ichii: Submitochondrial distribution of components of the steroid 11-\(\beta\)-hydroxylase and cholesterol sidechain-cleaving enzyme systems in hog adrenal cortex. J. Biochem. 65: 215–224 (1969).

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