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Nuclear Localization of Gold Labeled-hydrocortisone-bovine Serum Albumin Conjugate Injected Intravenously into the Hormone-target Cells of Rat

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ABSTRACT. **We have suggested in a previous study using 2-nm colloidal gold labeled-testosterone-bovine serum albumin (testosterone-BSA-gold) that 2-nm gold labeled-steroid hormone-BSA conjugates would be a useful tool for analyzing the mechanism of steroid hormone action (39). In this study, we examined whether hydrocortisone-BSA conjugate (hydrocortisone-BSA) showed a similar distribution to radiolabeled hydrocortisone *in vivo*, by injecting 2-nm colloidal gold labeled-hydrocortisone-BSA (hydrocortisone-BSA-gold) into the rat tail vein. The hydrocortisone-BSA-gold with silver enhancement became visible as silver deposits under electron microscopy in the nuclei of hepatocytes and hepatic stellate cells but not in Kupffer cells in the liver, and in the thymocytes and thymic reticuloepithelial cells in the thymus of a rat killed 2 h postinjection. The percentage of nuclei showing deposits in the non-target cells, the epithelial cells of the seminal vesicle, was similar to the value in the seminal vesicle of a control rat injected with BSA labeled with 2-nm colloidal gold as reported previously. In the hepatocytes and thymocytes of a control rat not injected, the percentages of nuclei showing deposits were similar to those in the rat injected with testosterone-BSA-gold or BSA-gold as reported previously, but lower than those in the rat injected with hydrocortisone-BSA-gold. These results suggest that hydrocortisone-BSA-gold is useful for the morphological study of hydrocortisone target cells, and imply that BSA conjugated with hydrocortisone can enter the target cell nuclei of the rat. The present study further indicates that the fate of gold labeled-steroid hormone-BSA conjugates may be decided at the cell membrane level.**

Key words: hydrocortisone-bovine serum albumin conjugate/target cell nuclei/colloidal gold/silver enhancement/in vivo/electron microscope

The steroid hormones circulate in blood plasma in three physical states: free, albumin-bound, and serum steroid binding protein-bound such as sex hormone-binding protein (SBP) and corticosteroid-binding globulin (CBG) (31, 43, 45, 52). Radiolabeled sex hormone (55), e.g. testosterone, and glucocorticoids (5, 8, 9, 41, 58, 62, 68), e.g. hydrocortisone, may bind to the hormone-target cell nuclei. According to the current model of steroid hormone action (26, 27), free nonprotein-bound hormones cross the cell membrane under passive transport to bind to the receptor in the cytoplasm, and the hormone-receptor complex is then translocated into the target cell nucleus. It has been reported that glucocorticoids enter the nucleus according to the free hormone hypothesis (4, 21, 35, 46). On the other hand, some researchers have questioned whether hydrophobic steroid hormones encounter no obstacle in penetrating the lipid bilayer of the cell membrane (1, 51, 65). Pietras and Szego have suggested that endocytotic vesicles appear to serve as vehicles for the nuclear transfer of steroid hormones (47).

Sex steroid hormone receptors are located only in the nucleus (14, 25, 29, 66). Testosterone coupled with SBP or with androgen-binding protein is internalized by a receptor-mediated endocytosis in the hormone-target cells, and then enters these cell nuclei *in vitro* (16–18). We have reported that testosterone-bovine serum albumin conjugate labeled with 2-nm colloidal gold (testosterone-BSA-gold) injected into the vascular system of rats becomes visible as silver deposits on the sections of tissues embedded in epoxy resin after silver enhance-

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Abbreviations: BSA, bovine serum albumin; CBG, corticosteroid-binding globulin; SBP, sex hormone-binding protein.

ment, and that testosterone-BSA-gold enters androgen-target cell nuclei, e.g. the epithelial cells of the seminal vesicle, but not non-target cell nuclei such as those of the liver and thymus (39). We have also demonstrated the possibility that testosterone-BSA-gold taken up in the vacuole by receptor-mediated endocytosis can enter the nucleoplasm by the fusion of the vacuole membrane with the partial diaphragm in the nuclear envelope, without passing through the cytosol (40).

Glucocorticoid binding sites are located on the cell membrane (1, 13, 23, 48, 53, 64) and the nuclear envelope (20, 28, 32, 34, 53) of the hormone-target cells, as well as on the sex steroid hormones (33, 34). CBG binds specifically to cell membranes (52, 61) and is found within the cells of glucocorticoid target tissues without degradation of the protein (30) and also within the nuclei of cultured cells (31, 52) and in the nuclear fraction of the liver (57). In an enucleation experiment, the unfilled receptor for glucocorticoids was not found in cytoplasm (67), as was the case with that of sex steroid hormone (66, 67). There are some reports that glucocorticoid receptors are located only in the nucleus in the presence or absence of the hormone (7, 36, 44, 49). Gasc *et al.* suggested for glucocorticoid action in the rat liver that no cytoplasm step would be required for nuclear translocation, as proposed for the sex steroid hormone (15). It is unlikely that macromolecules, e.g. glucocorticoid-BSA conjugate, freely traverse the cell membrane to enter the cytosol. If the binding of glucocorticoid with the cytoplasmic receptor is not essential for the nuclear translocation of the hormone, this would mean glucocorticoid-BSA conjugates can enter the hormone-target cell nuclei *in vivo*.

Corticosterone-succinate-BSA competes with [^{3}H]-corticosterone in its binding and uptake in isolated hepatocytes (63). The specific nuclear binding of glucocorticoids is detected in the nuclei of thymocytes of 10- or 12-week-old rats (9, 41). Hydrocortisone binds to both the cell membrane (48, 64) and the nuclei of hepatocytes (5, 58, 62), and to the nucleus in thymus (8, 58, 62, 68) of rat. Therefore, hydrocortisone-succinate-BSA was labeled with 2-nm gold colloid (hydrocortisone-BSA-gold) to study its uptake by the hormone-target cell nuclei of rats. In this study, we report on the localization of hydrocortisone-BSA-gold with silver enhancement in the liver and thymus as glucocorticoid target organs (5, 37, 41, 58, 62) and in the seminal vesicle as the non-target organ (3) under electron microscopy. The distribution of silver deposits implying the presence of hydrocortisone-BSA-gold is then compared with those implying testosterone-BSA-gold or 2-nm colloidal gold-labeled BSA (BSA-gold) in the three tissues reported previously (39).

Materials and Methods

Coupling of hydrocortisone-BSA and 2-nm colloidal gold

Hydrocortisone 21-hemisuccinate-bovine albumin conjugate (21 mols steroid per mole albumin, Sigma, St. Louis, MO, USA) was mixed with 2-nm colloidal gold solution (BioCell) according to the method described by Beppu (6), and then centrifuged as described previously (39). The pellet was dissolved in phosphate buffer (10 mM Na_2HPO_4, 3 mM KH_2PO_4, 123 mM NaCl, pH 7.4) containing 0.1% BSA, 0.05% polyethyleneglycol 20 M and 5% (v/v) glycerin. The concentration of hydrocortisone-BSA-gold was 0.5 mg/ml, and it was stored at 4°C until use.

Administration of hydrocortisone-BSA-gold to rat

A rat (Wistar strain, male, 10-weeks-old) was injected with 1-ml hydrocortisone-BSA-gold in the tail vein under ether anesthesia. After 2 h, the rat was fixed by perfusion of the glutaraldehyde fixative as described previously (39). The liver, thymus and seminal vesicle were removed, cut into small pieces and immersed in the same fixative for 2 h. The tissue pieces were washed with collidine buffer, postfixed in 1% OsO_4, dehydrated, and embedded in epoxy resin as described previously (39). Three to five resin-embedded tissue blocks per organ were used to make thin-sections of about 0.6 mm square. About 10 sheets of sections were mounted on nickel grids. In the liver, the tissue blocks were trimmed so that the midlobular zone of hepatic loble would be in the center of the thin-section.

Control experiment for silver enhancement

A second control rat, which received no injection, was fixed by perfusion as above. The liver and thymus were removed, cut into pieces, fixed and embedded as above.

Silver enhancement on epoxy resin

The sections were treated with 10% H_2O_2 for 10 min and then washed four times with distilled water containing 0.05% (v/v) Tween 20 for 10 min. The sections were reacted with a silver enhancement kit (BioCell) for 6 or 12 min at 4°C, washed thoroughly with distilled water containing Tween 20, then with distilled water only, dried, and stained with uranyl acetate. To assess the percentages of the nuclei showing silver deposits, all nuclei on the grids were examined under the electron microscope.

Results

In the rat sacrificed 2 h after injection of hydrocortisone-BSA-gold, silver deposits implying the presence of hydrocortisone-BSA-gold were found on the blood

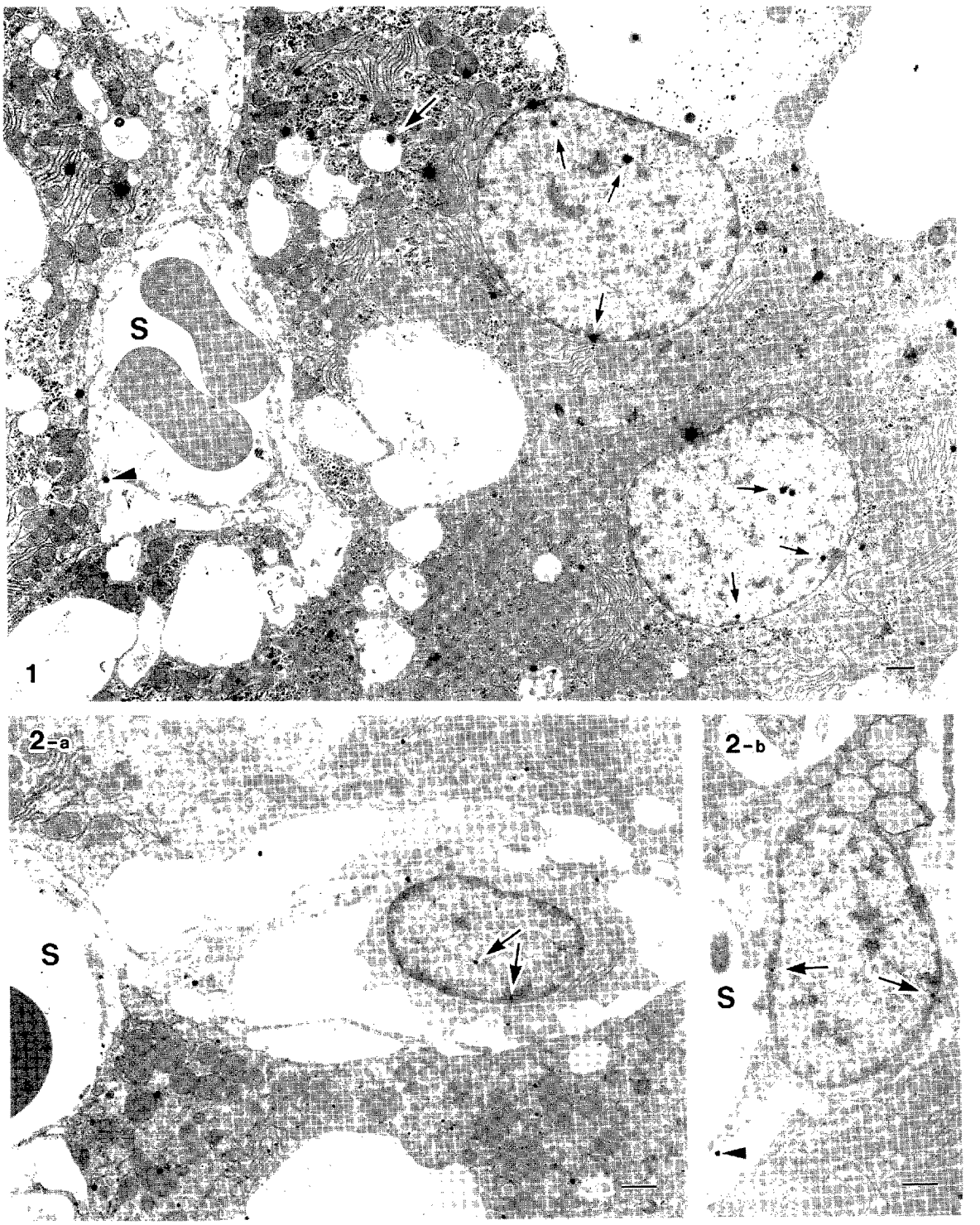

Fig. 1 and 2. Electron micrographs of liver of the rat sacrificed 2 h after injection of hydrocortisone-BSA-gold. Ultrathin sections were processed through silver enhancement for 6 min. Fig. 1. Silver deposits (arrows) are present on the nuclei or the nuclear envelope and the vacuole (large arrow) of hepatocytes. A deposit (arrowhead) is related to the cell membrane of hepatocyte. Bar = 1 μm. Fig. 2. Silver deposits (arrows) are present on the nuclei of hepatic stellate cells both without (a) and with lipid droplets (b). A deposit (arrowhead) is localized in the Disse's spaces (b). S, sinusoidal capillary. Bars = 1 μm.

plasma and Disse's spaces in the liver. In the hepatocytes (liver cells), several deposits were localized on the cytoplasm and the nucleus (Fig. 1). Some deposits seemed to be on the cell membrane and to be localized along the inner side of vacuoles. In the nucleus, the deposits seemed to be related to the nuclear envelope and were found on the nucleoplasm (Fig. 1). When the percentage of hepatocyte nuclei showing silver deposits was assessed under electron microscopy, about 13% of the ultrathin-sectioned nuclei revealed the presence of one to several silver deposits on the nuclei (Table I). The average number of deposits in the nucleus was 0.22.

In the nonparenchymal cells, the deposits were also found on the nuclei of the hepatic stellate cells (Ito cells) both with and without the lipid droplets in the section (Fig. 2). By contrast, in the nuclei of the Kupffer cells, few deposits were localized (Fig. 3). Only a few deposits were observed on the cytoplasm. The percentage of nuclei showing the deposits was also estimated in the hepatic stellate cells and Kupffer cells, their values being 23.9 ± 13.5 and 2.3 ± 1.5, respectively (Table I). In the cells examined in this study, the percentage of nuclei showing silver deposits was highest in the hepatic stellate cells.

In the thymus, silver deposits were found on the nuclei and the cytoplasm of the thymocytes (lymphocytes) and the thymic reticuloepithelial cells (Fig. 4). Almost all deposits were localized in the vicinity of or on the heterochromatin in the thymocyte nuclei (Fig. 4a). The percentage of the nuclei showing deposits was 6.2 ± 1.1 in the thymocytes (Table I). In the seminal vesicle, silver deposits were localized on the loose connective tissue. In epithelial cells of the seminal vesicle, a few deposits were found on the cytoplasm and the nuclei (Fig. 5). The percentage of the nuclei showing

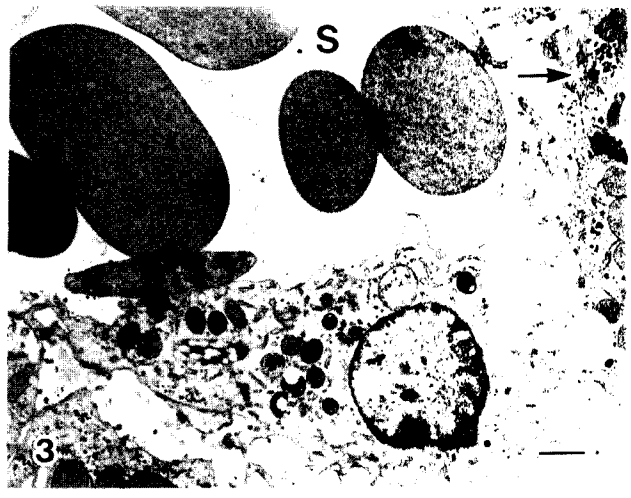

Fig. 3. Electron micrograph of liver of the same rat as in Figure 1. Ultrathin sections were processed through silver enhancement for 6 min. No silver deposit is seen on the Kupffer cell. A deposit (arrow) is on the hepatocyte. S, sinusoidal capillary. Bar = 1 μm.

Table I. DISTRIBUTION OF THE SILVER DEPOSITS ON THE NUCLEI IN THE TISSUES 2 H AFTER INJECTION OF STEROID HORMONE-BSA-GOLD OR BSA-GOLD OR IN CONTROL

Tissue	Cell	Treatment	Total No. of nuclei[1]	No. of nuclei showing silver deposits (% of total ± SD)[2]
Liver	Hepatocyte	hydrocortisone-BSA-gold	1,801	235 (13.0 ± 2.1)
		testosterone-BSA-gold*	1,385	22 (1.6 ± 1.0)
		BSA-gold*	770	10 (1.3 ± 1.2)
		none	1,493	35 (2.3 ± 1.1)
	Hepatic stellate cell	hydrocortisone-BSA-gold	71	17 (23.9 ± 13.5)
	Kupffer cell	hydrocortisone-BSA-gold	132	3 (2.3 ± 1.5)
Thymus	Thymocyte	hydrocortisone-BSA-gold	12,028	743 (6.2 ± 1.1)
		testosterone-BSA-gold*	4,985	48 (1.0 ± 0.3)
		BSA-gold*	6,046	37 (0.6 ± 0.4)
		none	6,685	65 (1.0 ± 0.8)
Seminal vesicle	Epithelial cell	hydrocortisone-BSA-gold	2,927	202 (6.9 ± 1.5)
		testosterone-BSA-gold*	2,154	671 (31.2 ± 1.6)
		BSA-gold*	2,473	124 (5.0 ± 0.6)
		none*	1,111	5 (0.5 ± 0.04)

[1] Total of nuclei counted in ultrathin sections.
[2] Number of nuclei showing one to several deposits. Values are means ± standard deviation.
* Data reported previously (39).

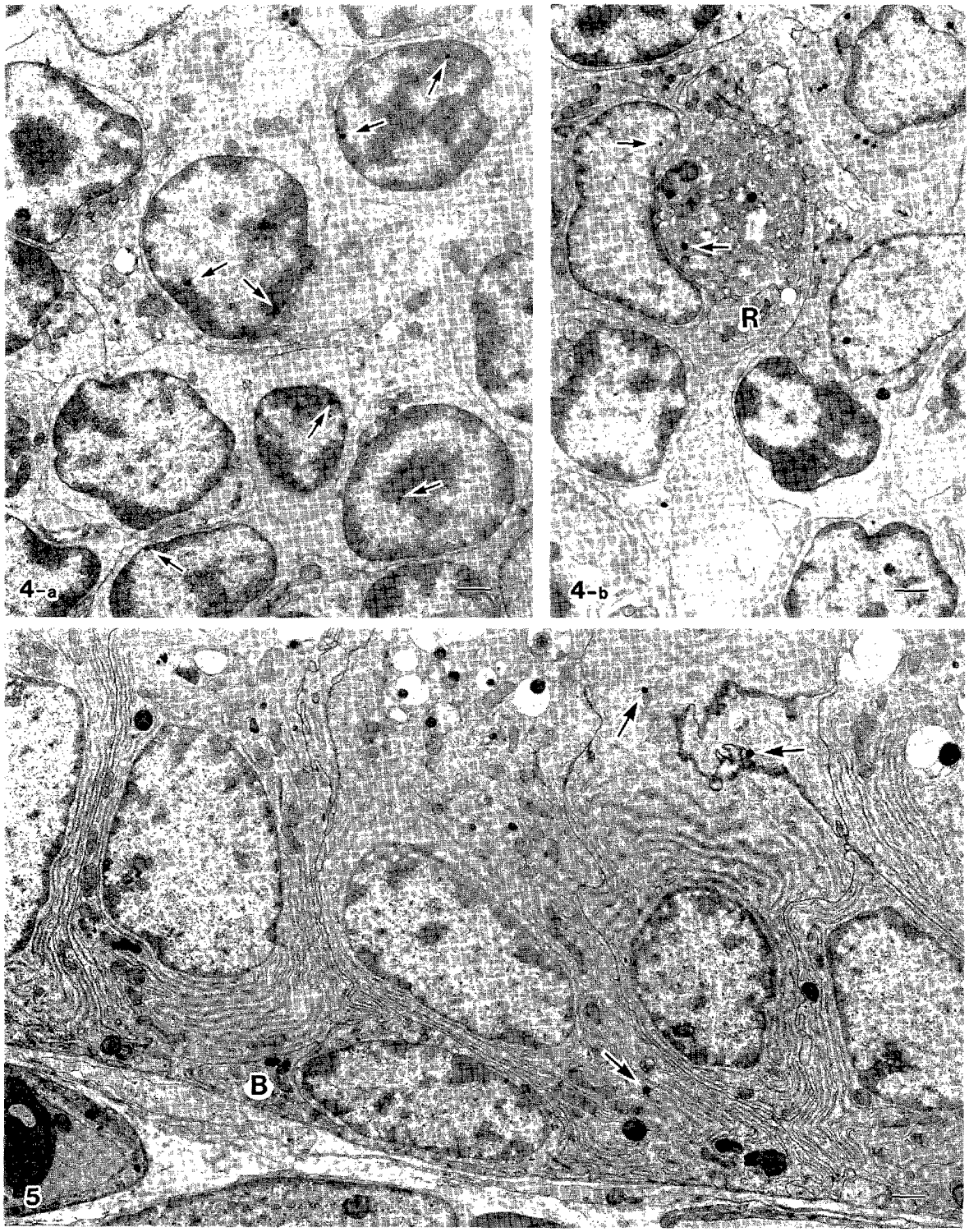

Fig. 4 and 5. Electron micrographs of thymus and seminal vesicle of the same rat as in Fig. 1. Ultrathin sections were processed through silver enhancement for 6 min (Fig. 4) or 12 min (Fig. 5). Fig. 4. (a) Silver deposits (arrows) are present on the nuclei of thymocytes. (b) Silver deposits (arrows) are found on the nucleus and the cytoplasm of the thymic reticuloepithelial cell (R). Bars=1 μm. Fig. 5. A few silver deposits (arrows) are present on the cytoplasm of epithelium, but not on the nuclei of the epithelium or the basal cell (B). Bar=1 μm.

deposits was 6.9±1.5 in the epithelial cells of seminal vesicles (Table I).

When the liver and the thymus of the uninjectd control rat were processed by silver enhancement, very few deposits were found on these tissues. The percentages of the nuclei showing deposits were 2.3±1.1 in the liver cells and 1.0±0.8 in the thymocytes (Table I). The average number of deposits in the nucleus was 0.03 in hepatocytes.

Discussion

There are many studies based upon the use of ultrasmall gold (1–1.4 nm) labeled antibodies in combination with silver enhancement (22, 59, 60). Gold particles embedded in epoxy resin increase their size after controlled silver enhancement (10, 39). In the rat injected with testosterone-BSA-gold, we have reported that the silver deposits implying the presence of testosterone-BSA-gold in the hormone-target tissues are observed at the same location as the radioactivity in the rats administrated [^{3}H]-testosterone, but are few on the hepatocyte nuclei (39). This study of the hepatocytes shows that the percentage of nuclei showing the deposits implying the presence of hydrocortisone-BSA-gold is higher than that in the rat injected with testosterone-BSA-gold, with BSA-gold or nothing (Table I). These results suggest that hydrocortisone-BSA-gold is internalized by the hepatocytes and transported to the nuclei, and are consistent with a report of nuclear concentration of hydrocortisone radioactivity in the hepatocytes (58), and are supported by a report that nuclear receptors in the rat liver are specific for hydrocortisone, not testosterone (58).

A synthetic glucocorticoid, dexamethasone, affects the gene expression of hepatic stellate cells isolated from rat liver (50). Our result in the hepatic stellate cells is corroborated by a report that the immunoreactivity of glucocorticoid receptor is present in the cell nuclei (49). It is unclear why the hepatic stellate cells show the highest value in the percentage of nucleus showing silver deposits.

Kupffer cells take up some foreign substances injected into the rat. Our result in Kupffer cells is consistent with our previous result that testosterone-BSA-gold is not taken up by macrophages until 2 h after injection (39), and agrees with a report that the immunoreactivity of glucocorticoid receptor is absent in Kupffer cells (2). The observed distribution, however, is incompatible with a report that Kupffer cells show the immunoreactivity of glucocorticoid receptor (49). Further study is necessary to determine whether or not hydrocortisone-BSA-gold enters the Kupffer cell nuclei.

Effects of glucocorticoids on the thymus are well known *in vivo* (11, 38). Glucocorticoid receptor is detected in the nuclei of thymocytes and reticuloepithelial cells (37, 42). This study shows that the localization of hydrocortisone-BSA-gold is consistent with that of the receptor, and that the percentage of thymocyte nuclei showing the existence of hydrocortisone-BSA-gold is higher than that of testosterone-BSA-gold, BSA-gold or nothing. These results suggest that hydrocortisone-BSA-gold enters the nuclei of thymocytes, and is supported by reports that nuclei of the thymus combine with hydrocortisone, not testosterone (58), and that no androgen receptor is localized in thymocytes (19, 54).

We have suggested that testosterone-BSA-gold enters the epithelial cell nuclei of the seminal vesicle, as does radiolabeled testosterone (39). In the seminal vesicle of a rat injected with hydrocortisone-BSA-gold, the percentage of epithelial cell nuclei showing the existence of hydrocortisone-BSA-gold is similar to that in the control rat injected with BSA-gold. This result is supported by a report that the seminal vesicle is a nontarget organ for glucocorticoids (3). However, it is unclear why the percentages in both hydrocortisone-BSA-gold and BSA-gold are higher than that of rat injected with nothing. By contrast, the effects of hydrocortisone on the epithelial cells of male accessory sexual organs have been indicated in organ cultured rat prostate (24). Glucocorticoid receptor-like immunoreactivity is localized within the basal cell nuclei of the ductal wall and in the fibroblast nuclei of the connective tissue in male rat accessory sexual organs such as the seminal vesicle (56). Therefore, further study is needed to determine the distribution of glucocorticoids in male accessory sexual organs.

Gold labeled-corticosterone-succinate-BSA binds to the cell membrane of isolated hepatocytes (63). Hydrocortisone binds to specific sites in the cell membrane of hepatocytes (64). Testosterone-BSA-gold is taken up by receptor-mediated endocytosis in the hormone target cells and enters the nuclei (40). This study shows that silver deposits implying the presence of hydrocortisone-BSA-gold are localized on the cell membrane, the vacuoles, the nuclear envelope and the nucleoplasm of the hormone-target cells, as are the deposits of testosterone-BSA-gold. The results suggest that hydrocortisone-BSA-gold enters the nucleus in a way similar to that in the nuclear translocation of testosterone-BSA-gold.

BSA labeled with 5-nm colloidal gold is taken up by the isolated hepatocytes and accumulated in their lysosomes (12). Hepatocytes are metabolic cells for steroid hormone (45). Testosterone-BSA-gold is taken up by the hepatocytes (39), but does not enter their nuclei. As stated above, hydrocortisone-BSA-gold is transported to the hepatocyte nuclei. Similarly, hy-

drocortisone-BSA-gold enters the thymocyte nuclei, but testosterone-BSA-gold does not. In contrast, testosterone-BSA-gold enters the nuclei of epithelial cells in the seminal vesicle, as reported previously (39). It is unlikely that macromolecules, such as hydrocortisone-BSA-gold, pass freely through the cell membrane to enter the cytosol. Consequently, the present study indicates that whether or not steroid-BSA-gold is transferred to the nucleus may be decided at the cell membrane level, and suggests that the binding of hydrocortisone-BSA-gold with the cytoplasmic receptor may not be essential for its nuclear translocation.

In conclusion, hydrocortisone-BSA-gold can enter the nuclei of the glucocorticoid-target cells, as testosterone-BSA-gold does in the nuclei of testosterone-target cells. These findings confirm our proposal in previous studies (39, 40) that 2-nm gold labeled-steroid hormone-BSA conjugates are a useful tool for analyzing the mechanism of steroid hormone action, and indicate the possibility that both glucocorticoids and sex steroid hormones might be used as carriers of foreign macromolecules, e.g. proteins, into the target cell nuclei *in vivo*.

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