IMMUNOPEROXIDASE STAINS CORTISOL IN ADRENAL AND PITUITARY

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The unlabeled peroxidase-anti-peroxidase (PAP) method of Sternberger was used to localize cortisol within paraffin embedded sections of cat adrenal and pituitary tissue. Incubation of the cortisol antiserum used in this method with increasing concentrations of cortisol led to progressive extinction of cortisol staining of the adrenal fasciculata cells, (as measured with a scanning integrating microdensitometer). This result suggests strongly that the staining achieved with this method was specific for cortisol. Cortisol staining was demonstrated not only within cells that synthesize cortisol (the adrenal fasciculata) but also in cells of the adrenal medulla and of the anterior pituitary, two target sites for cortisol action.

A variety of hormones (3) and hypothalamic releasing factors (6) have been demonstrated both by light and by electron microscopy with the use of the peroxidase-anti-peroxidase (PAP) method of Sternberger (7). However, this immunohistochemical technique has not hitherto been reported for visualizing a steroid moiety or hormone. We were interested in using this highly sensitive (4) and selective method of staining to localize cortisol both in cortisol synthesizing cells and in cells at cortisol target sites.

Cortisol is known to inhibit the release of ACTH from the pituitary (1). Cortisol also acts on cells of the adrenal medulla to induce an enzyme phenylethanolamine-N-methyltransferase (9). We were interested in seeing whether or not immunohistochemical techniques would be sensitive enough to demonstrate the presence of cortisol within these cells, since if this was determined, immunohistochemistry might provide an alternative and more precise method for studying cortisol uptake, within such tissues as brain, than the autoradiographic (8) and radioisotopic (2) techniques used to date.

MATERIALS AND METHODS

Adult cats of either sex (2.0–2.5 kg) were used for these studies and were allowed to eat and drink ad libitum. Adrenal glands were removed from cats anesthetized with chloroform and perfused through the heart with cold phosphate buffered saline (PBS 0.1 M), pH 7.4. Adrenal glands were fixed by immersion in Bouin’s solution for 48 hr, and dehydrated in graded ethanol’s and cleared in xylene before embedding in paraffin. Sections were cut at 4 μm and mounted on glass slides coated with gelatin and egg albumin dried overnight at 60°C. Pituitary glands were removed from cats given a 100-mg intravenous injection of hydrocortisone 1 hr previously. The cats were anesthetized with chloroform and perfused through the left ventricle with 250 ml of PBS (0.1 M), pH 7.4, followed by 250 ml of Bouin’s solution. The pituitary was immersed in Bouin’s and processed as the adrenal tissue described above.

The cortisol antiserum used was prepared in our own laboratory from rabbits hyperimmunized with cortisol-21-hemisuccinate conjugated to bovine thyroglobulin (5). The antiserum had a titer of between 1:30,000 and 1:50,000. The cross-reactivity of the antiserum (measured at 50% displacement of 1H-cortisol) of various steroids is shown in Table 1. The rabbit ACTH antiserum had a titer of 1:30,000 and 1:50,000. The cross-reactivity of the antiserum (measured at 50% displacement of 1H-cortisol) of various steroids is shown in Table 1. The rabbit ACTH antiserum had a titer of 1:10,000, crossreacted completely only with 1–39 ACTH, and did not crossreact with growth hormone, prolactin, thyroid stimulating hormone or leutinizing hormone.

TABLE 1

<table>
<thead>
<tr>
<th>Steroid</th>
<th>% Cross Reacting</th>
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<tbody>
<tr>
<td>Cortisol</td>
<td>100</td>
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<tr>
<td>11-Deoxycortisol</td>
<td>33</td>
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<tr>
<td>Cortisone</td>
<td>25</td>
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<tr>
<td>Corticosterone</td>
<td>22</td>
</tr>
<tr>
<td>17-hydroxyprogesterone</td>
<td>12</td>
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<tr>
<td>Testosterone</td>
<td>8</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>1</td>
</tr>
<tr>
<td>Aldosterone</td>
<td>0.2</td>
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1 Supported in part by National Institutes of Health grants AM07109 and AM14952, and by the Camilla Samuel Fund.
Fig. 1. A, adrenal gland of the cat (×40) showing immunostaining for cortisol in the fasciculata layer (b) but not in the outer glomerulosa (a) or in the zona reticularis (c). B, adrenal medulla (×100) showing both cytoplasmic and nuclear immunostaining for cortisol. C, adrenal gland of the dog (×40) showing both cytoplasmic and nuclear immunostaining for cortisol. D, adrenal fasciculata cells (×400) in which normal rabbit serum (1:10) was substituted for the cortisol antisem as a control.
Adrenal and pituitary tissue were stained by the PAP method as follows. Slides were deparaffinized and rehydrated in 0.01 M PBS, pH 7.4. Sequential application to slides of 5% of the following antisera were made: 1) Cortisol or ACTH antiserum diluted 1:150 in PBS; 2) Goat antiserum rabbit (GAR, Cappel Labs, Downingtown, Pa.) immunoglobulin (IgG) diluted 1:50 in equal volumes of PBS and 10% normal goat serum; 3) PAP, with the anti-peroxidase being made in rabbit (Cappel) similarly diluted 1:50 as for GAR. The slides were incubated with each of the antisera for 30 min at room temperature in a moist chamber. The slides were washed between each incubation in three 5-min washes of PBS in an agitated bath. Between the cortisol and GAR incubation, the slides were incubated for 10 min with 10% goat serum to reduce nonspecific background staining. After the PAP incubation and wash, the slides were transferred to a jar containing a fresh solution of 0.2% diaminobenzidine (DAB, Sigma, St. Louis, Mo.) and 0.003% hydrogen peroxide diluted with tris buffer (Sigma) 0.05 M, pH 7.5. The slides were then washed in TRIS before counterstaining with methyl-green, dehydrating through the alcohols and coverslipping with Permount. As controls, sera consisting of either: 1) normal rabbit serum; or 2) rabbit antiserum to calcitonin and insulin; or 3) cortisol antiserum absorbed with added cortisol were substituted for the cortisol antiserum. Absorption of cortisol antiserum by cortisol was quantified by scanning densitometry. Cortisol antiserum was incubated for 72 hr at 4°C with increasing concentrations of cortisol. The final dilution of antiserum was kept at 1:150. The final concentration of alcohol being used to dissolve the cortisol was kept at 2%, since at this concentration of alcohol there was no quantitative effect on immunostaining. When the final alcohol concentration was >10%, a diminution of immunostaining was seen. The cortisol antiserum was then used to stain for cortisol in the adrenal, using exactly the same steps as described above. The final staining was quantified with the use of a Vickers scanning integrating microdensitometer, set at wave length 460 nm. Each adrenal slide had 10 individual cells from the zona fasciculata counted; the scanning mask covered only one cell at a time. A mean density for each slide was computed as the average raw density minus the density for a control slide in which rabbit serum replaced cortisol antiserum in the method. The mean density (and the s.e.m.) was then expressed as a percentage of the maximum density seen with cortisol antiserum alone.

**RESULTS**

Immunostaining for cortisol was seen as a dark brown granular reaction product by light microscopy. In the adrenal, both the cytoplasm and the nuclei of cells in the zona fasciculata were stained (Figs. 1A,B). The nuclei of cells of the zona reticularis and of the adrenal medulla were also positive (Fig. 1C). Control slides showed no dark brown granular staining in any region (Fig. 1D) although some non-specific staining was seen around the adrenal capsule and in blood vessels. No staining was seen in adrenal cells themselves. The counterstain methyl-green depicts the nuclei in the control slides as a darker green than the cytoplasm.
making them clearly visible; however, these nuclei showed no immunostaining.

Immunostaining of the adrenal fasciculata was diminished by prior incubation of cortisol antiserum with cortisol. The percentage of maximal immunostaining of the adrenal fasciculata cells (quantitated by scanning integrating microdensitometry) is plotted with its s.e. against the amount of cortisol added in Figure 2. The total extinction of immunostaining after incubation with 1 mg of cortisol is shown in Figure 3.

Serial sections of the pituitary gland were stained with ACTH and cortisol antisera. Cortisol staining was present in the same cells of the anterior pituitary that also showed immunostaining with ACTH (Fig. 4A,B). No immunostaining was seen when other hyperimmune or preimmune sera were used (Fig. 4C).

Fig. 3. Adrenal fasciculata cells (×400) showing no immunostaining for cortisol after the cortisol antiserum was incubated for 72 hr at 4°C with 1 mg of cortisol.

Fig. 4. A, anterior pituitary gland of the cat (×400) showing positive immunostaining for ACTH. B, an adjacent section of anterior pituitary gland (×400) showing immunostaining for cortisol in the same cells. C, no immunostaining was seen when normal rabbit serum was substituted for the specific antiserum in any pituitary cells.
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DISCUSSION

We have shown that the unlabeled PAP method of Sternberger (7) can be used to visualize cortisol in paraffin embedded tissue. The strong positive staining of cortisol in the adrenal zona fasciculata cells (the known site of cortisol synthesis) shows that the fixation, dehydrating and the paraffin embedding processes have not totally removed the cortisol present. It is possible that some of the cortisol did diffuse out of the tissue or from a nuclear to a cytoplasmic location during processing, since one would expect a steroid to be soluble in alcohol. Obviously however, enough cortisol remained in the fasciculata cells to stain consistently with the PAP method.

The question of specificity of this immunohistochemical technique naturally arises. That the staining for cortisol is specific is suggested strongly by the following: the staining of tissue known to contain cortisol (zona fasciculata); absence of staining in tissue that contains other steroids but not cortisol (zona glomerulosa); absence of staining with other antisera; and progressive extinction of staining by prior incubation with progressive amounts of cortisol.

The presence of cortisol within the nuclei of the adrenal medulla is not unexpected since this is a known site of cortisol action. It has been shown that induction of the enzyme phenylethanolamine-N-methyltransferase, the enzyme that converts noradrenaline to adrenaline in the adrenal, depends in part on cortisol (9).

The presence of cortisol immunostaining in cells of the anterior pituitary gland that stain strongly for ACTH suggests that this method of staining is sensitive enough to identify cortisol at a site of physiological action remote from the adrenal gland. Cortisol is known to exert a negative feedback effect on the pituitary gland to limit release of ACTH from the corticotropes (1).

Since the PAP method described is sensitive enough to stain for cortisol in target tissue as well as at sites of cortisol synthesis, immunohistochemistry may provide a powerful tool for looking at sites of cortisol uptake, and possibly feedback, in the brain. Until now, autoradiographic (8) and radioisotopic (2) techniques have provided the only means to study this question. Neither can provide the precise morphological detail that is inherent in the PAP method.

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LITERATURE CITED