

## Brief Report

## ABERRANT INTERLEUKIN-1 RECEPTORS IN A CORTISOL-SECRETING ADRENAL ADENOMA CAUSING CUSHING'S SYNDROME

HOLGER S. WILLENBERG, M.D.,  
 CONSTANTINE A. STRATAKIS, M.D., CHRISTIAN MARX,  
 MONIKA EHRHART-BORNSTEIN, PH.D.,  
 GEORGE P. CHROUSOS, M.D.,  
 AND STEFAN R. BORNSTEIN, M.D.

**C**ORTISOL-SECRETING adrenal adenomas are an uncommon cause of Cushing's syndrome. Little is known about the events leading to the formation of these tumors, but molecular defects, including activating mutations of receptors for corticotropic factors, have been suspected in this process. Structural mutations of the corticotropin-receptor gene have not been detected in these tumors,<sup>1</sup> but some have had gastric inhibitory polypeptide,<sup>2,3</sup> vasopressin,<sup>4</sup> and more recently,  $\beta$ -adrenergic receptors.<sup>5</sup>

In this report, we provide evidence of the involvement of immune cells and one of their cytokine products in the formation of an adrenocortical adenoma in a patient with Cushing's syndrome. A striking lymphocytic infiltration of the patient's adrenocortical adenoma led us to examine the effects of interleukin-1 on the synthesis of cortisol by the adenoma cells and the expression of type I and II interleukin-1 receptors on these cells. Nearly all the biologic activity of interleukin-1 can be accounted for by type I receptors. Type II receptors are not signal-transducing but are probably cleaved enzymatically to yield soluble interleukin-1-binding protein, which inhibits interleukin-1 activity.<sup>6,7</sup> We found that interleukin-1, but not corticotropin, increased the secretion of cortisol by the adenoma cells *in vitro*. In addition, type I interleukin-1-receptor protein and its messenger RNA (mRNA) were detected in the tumor cells.

From the Department of Internal Medicine III, University of Leipzig, Leipzig, Germany (H.S.W., C.M.); and the National Institute of Child Health and Human Development (H.S.W., C.A.S., G.P.C., S.R.B.) and the National Institute of Mental Health (M.E.-B.), National Institutes of Health Clinical Center, Bethesda, Md. Address reprint requests to Dr. Bornstein at the National Institutes of Health, NIH Clinical Center, NICHD, Bldg. 10, Rm. 10N262, Bethesda, MD 20892.

©1998, Massachusetts Medical Society.

### CASE REPORT

A 62-year-old woman was referred because of weight gain, hypertension, and hirsutism of three years' duration. Physical examination revealed an obese woman who weighed 75 kg and was 155 cm tall. Her resting pulse was 68 beats per minute, and her blood pressure was 160/95 mm Hg. She had facial plethora, hirsutism, telangiectasia, and ankle edema. The plasma cortisol concentration was 25  $\mu\text{g}$  per deciliter (690 nmol per liter; normal, 7.2 to 18.1  $\mu\text{g}$  per deciliter [200 to 500 nmol per liter]) in the morning and 19.8  $\mu\text{g}$  per deciliter (547 nmol per liter) in the evening, and urinary cortisol excretion was 129  $\mu\text{g}$  per day (357 nmol per day; normal, 7 to 36  $\mu\text{g}$  per day [20 to 100 nmol per day]). The morning plasma corticotropin concentration was undetectable (<4.5 pg per milliliter [1.0 pmol per liter]; normal, 9 to 52 pg per milliliter [2 to 11 pmol per liter]). The plasma and urinary cortisol values did not decrease in response to dexamethasone. Computed tomography revealed a 2-cm mass in the right adrenal gland that was removed surgically, with no complications. The hypercortisolism and stigmata of Cushing's syndrome resolved after the removal of the tumor.

### METHODS

#### Tissue Processing

Tissue from the patient's tumor and from cortisol-producing adenomas from four other patients with corticotropin-independent Cushing's syndrome was transferred to prechilled phosphate-buffered saline, pH 7.6, and kept on ice until immunohistochemical studies (four tumors) and *in vitro* studies (two tumors) were performed. Tissue from three patients with adrenal carcinoma was also studied, along with normal adrenal glands from four patients who underwent nephrectomy for renal carcinoma.

#### Immunohistochemical Analysis

Adrenocortical, chromaffin, and immune cells were characterized immunohistochemically in paraffin-embedded sections of adrenal tissue with antibodies to chromogranin A (clone DAK-A3, Dako, Hamburg, Germany) and 17 $\alpha$ -hydroxylase (courtesy of M.R. Waterman, Nashville), CD45 (clones 2B11 and PD7/26, Dako), and CD68 (clone KP1, Dako), as previously described.<sup>8</sup> The presence of interleukin-1-receptor protein was evaluated by immunostaining with two mouse monoclonal antibodies to type I interleukin-1 receptors (clone C-20, Santa Cruz, Heidelberg, Germany, and Genzyme, Cambridge, Mass.). Bound antibodies were detected by the linked streptavidin-biotin-peroxidase method (Dako), and the enzyme reaction was visualized with 3-amino-ethylcarbazole (Dianova, Hamburg, Germany). Monoclonal mouse immunoglobulin was used as a negative control.

#### In Situ Hybridization

Single-stranded DNA antisense probes were amplified with the polymerase chain reaction (PCR) with 3' primers for type I or type II interleukin-1 receptors.<sup>9</sup> Control sense probes were designed in the same manner, with 5' primers. All probes were purified with MicroSpin S-300 HR columns (Pharmacia Biotech, Heidelberg, Germany) and labeled with use of a reaction in which deoxyadenosine triphosphate and digoxigenin-deoxyuracil triphosphate were added to the 3' ends (Boehringer Mannheim, Mannheim, Germany). The digoxigenin-labeled probes were hybridized to cryostat-fixed sections of adrenal tumor tissue, and tissue-bound probe was stained with a monoclonal antibody to digoxigenin (Boehringer Mannheim). Sections of normal human adrenal tissue served as controls.

#### Isolation of RNA and Reverse-Transcription PCR

Total RNA was isolated from 5 million cells with the RNeasy Miniprep Kit (Qiagen, Hilden, Germany). Complementary DNA (cDNA) was synthesized by reverse transcription (Ready-To-Go T-primed first-strand synthesis kit, Pharmacia Biotech) after treat-

ment with deoxyribonuclease I (Boehringer Mannheim); cDNA for either type I or type II interleukin-1 receptor was amplified as previously described.<sup>9</sup> The PCR products of the type I and type II interleukin-1 receptors were verified by digestion with *Mbo*II and *Msp*I or *Mbo*II and *Eco*RI (Promega, Heidelberg, Germany), respectively. As a negative control, genomic DNA or RNA that was not reverse transcribed was processed in parallel.

#### Extraction of DNA and Analysis of Clonality

DNA was extracted from frozen tumor tissue and peripheral-blood lymphocytes from the patient as previously described.<sup>10</sup> The human androgen-receptor gene locus A (*HUMARA*, GenBank accession number M21748) was used to identify the clonal origin of the adenoma.<sup>11</sup>

#### Cell-Culture Experiments

Specimens of adrenal tumors and normal adrenal tissue were mechanically dissected and digested enzymatically. After being depleted of macrophages with a monoclonal mouse antibody to CD68 (KP-1, Dako) and sheep antimouse immunoglobulin Dynabeads M-450 (Dynal, Oslo, Norway),<sup>12</sup> the isolated cells were cultured in quadruplicate in 24-well plates (100,000 cells per well) for three days, as described previously.<sup>13</sup> The cells were then washed and incubated with  $10^{-8}$  M to  $10^{-12}$  M human interleukin-1 $\beta$  (Endogen, Cambridge, Mass.) or  $10^{-9}$  M corticotropin (Synacthen, Ciba-Geigy, Wehr, Germany) in serum-free medium for 6, 12, or 24 hours before cortisol concentrations were measured by radioimmunoassay (Biermann, Bad Nauheim, Germany).

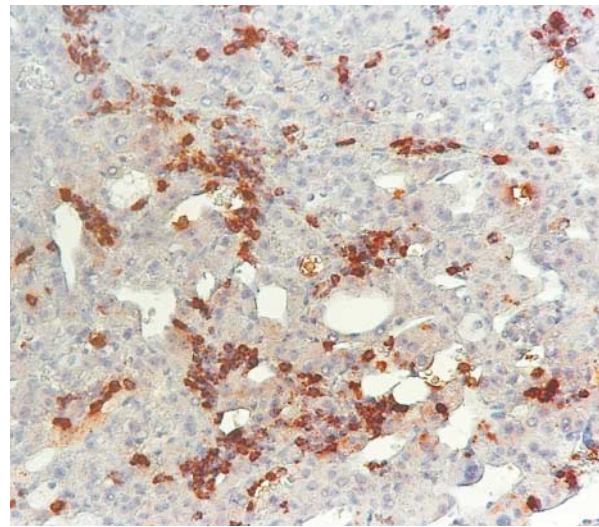
## RESULTS

#### Histologic Examination

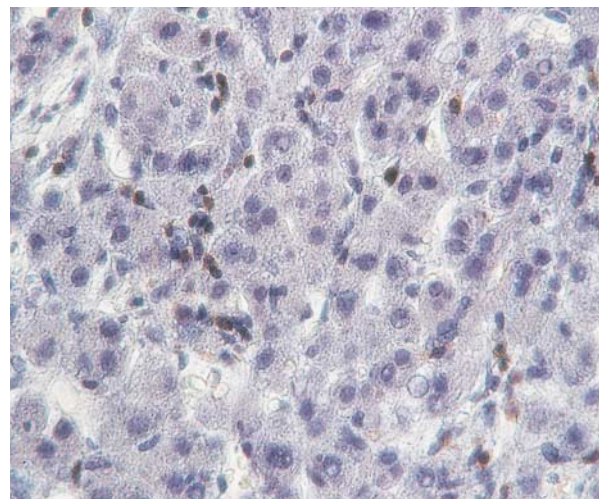
Microscopical examination of the patient's adrenal adenoma revealed a well-vascularized nodule that contained adenoma cells with no signs of malignancy and was heavily infiltrated by leukocytes.

#### Immunohistochemical Analysis

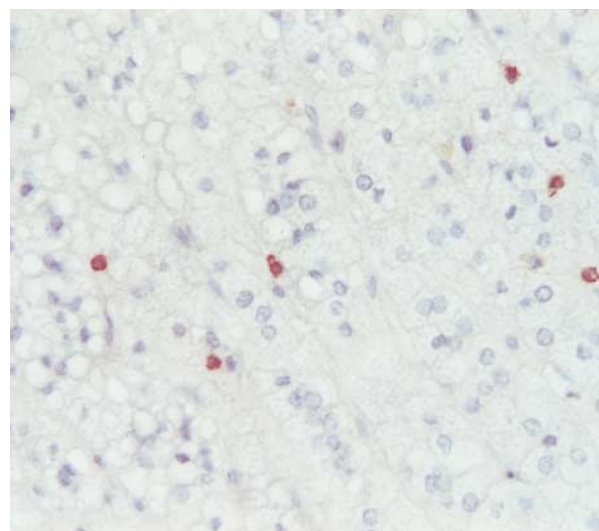
The patient's adenoma contained many cells that stained with antibodies to macrophage-specific CD45 and CD68 antigens, which are known to be a major source of interleukin-1 (Fig. 1A). In contrast, few cells stained with these antibodies in the other four specimens of adrenal adenoma, the three samples of adrenal carcinoma, or the four samples of normal adrenal gland (Fig. 1B and 1C). Specimens from the patient's adenoma stained intensely with antibodies



A



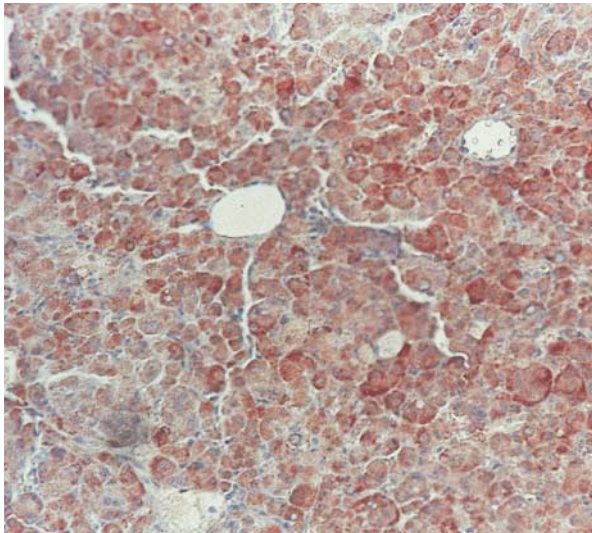
B



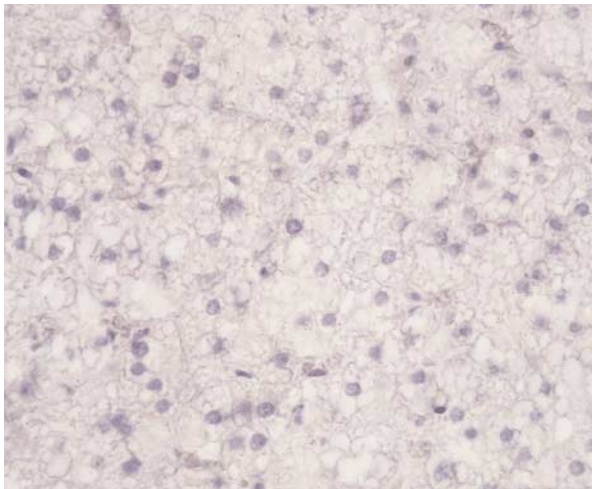
C

**Figure 1.** Immunostaining of Specimens of Adrenal Adenoma and Normal Adrenal Gland.

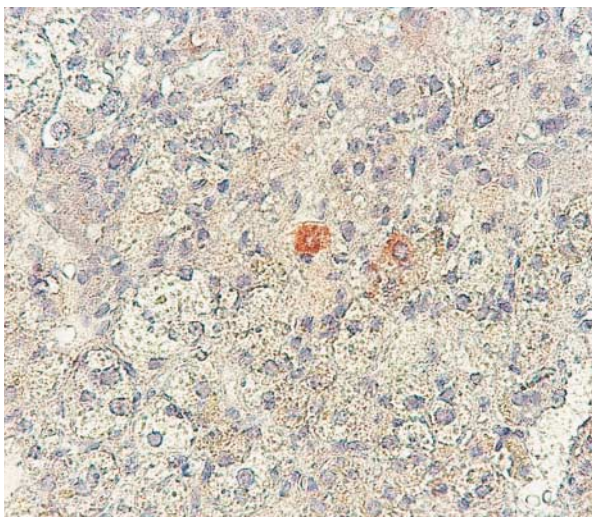
Panel A shows intense infiltration of the patient's tumor tissue by leukocytes immunostained red with antibodies to CD45 (clone 2B11) ( $\times 200$ ). In contrast, there were only a few CD45+ immune cells scattered throughout a representative section of an adrenal adenoma from another patient (Panel B,  $\times 400$ ) and in the cortex of a normal adrenal gland (Panel C,  $\times 400$ ). In Panel D ( $\times 200$ ), the patient's adrenal adenoma expresses type I interleukin-1 receptors (clone C-20) (red staining). In contrast, interleukin-1 receptors are not expressed in a representative section of an adrenal adenoma from another patient (Panel E,  $\times 400$ ) and are expressed only in immune cells in the cortex of a normal adrenal gland (Panel F,  $\times 400$ ). The reaction was visualized with 3-amino-ethylcarbazole.



D



E



F

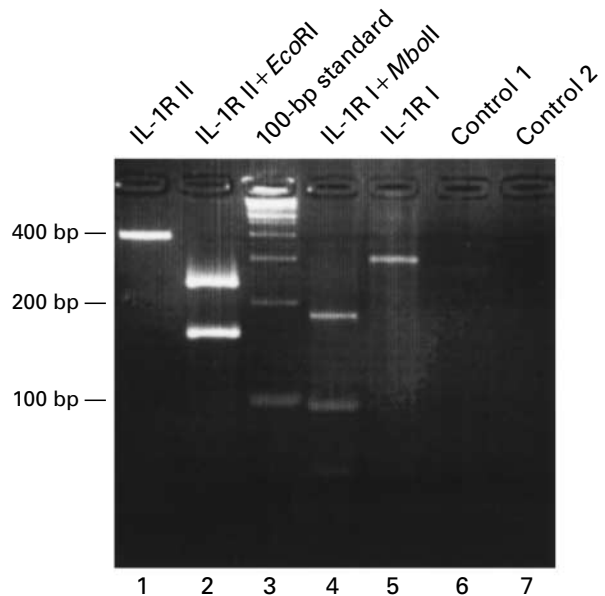
to type I interleukin-1 receptor (Fig. 1D), whereas the tissues from the other adrenal adenomas and carcinomas and normal adrenal tissue did not stain with this antibody (Fig. 1E and 1F).

#### In Situ Hybridization

A strong signal indicating the presence of type I interleukin-1-receptor mRNA was detected throughout the patient's adrenal adenoma but not in normal adrenal tissue. In contrast, signals indicating the presence of type II interleukin-1 receptor mRNA were detected in perivascular regions of all samples of adrenal gland (data not shown).

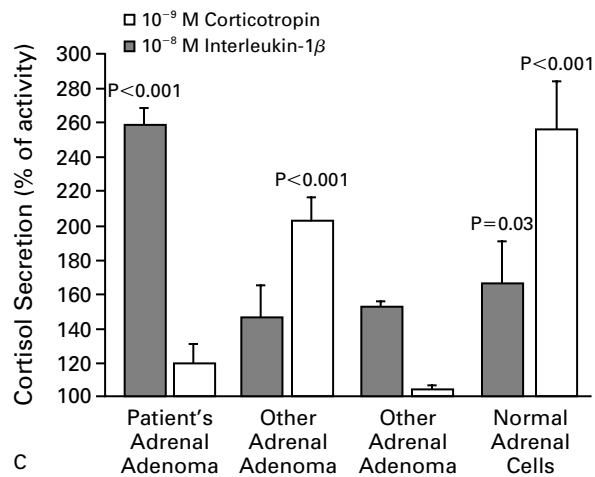
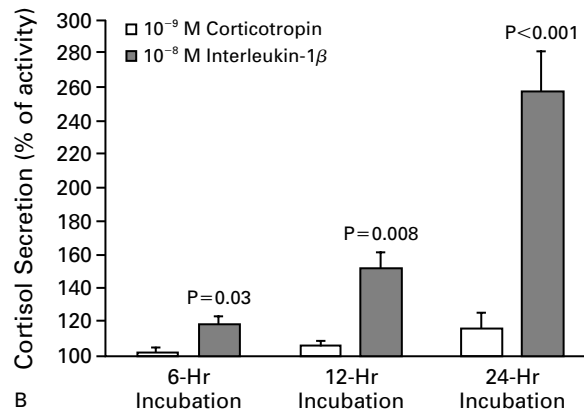
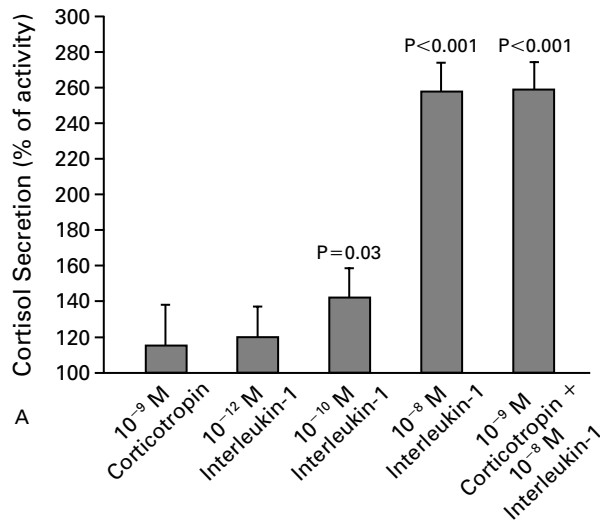
#### Reverse-Transcription PCR

A reverse-transcription-PCR assay for the interleukin-1-receptor mRNA in cells isolated from the patient's adrenal tumor yielded the expected cDNA fragments of 299 bp for type I receptors and 391 bp for type II receptors, and enzymatic digestion of the PCR products confirmed the results (Fig. 2). The results of this assay were negative in cells isolated from normal adrenal glands and the other adrenal



**Figure 2.** Reverse-Transcription-PCR Assay for Type I and Type II Interleukin-1 Receptors (IL-1R) in Messenger RNA from the Patient's Adrenal Adenoma Cells.

Total RNA was isolated, and cDNA was synthesized by reverse transcription and identified as coding for type I or type II interleukin-1 receptors. The results of the assay were confirmed by enzymatic digestion of the PCR products. Lane 1 shows a 391-bp fragment confirmed by digestion with *EcoRI* (lane 2) to be type II interleukin-1-receptor mRNA. Lane 3 shows the 100-bp standard. Lane 5 shows a 299-bp fragment confirmed by digestion with *MboII* (lane 4) to be type I interleukin-1-receptor mRNA. Lane 6 shows a negative control in which no template was used. Lane 7 shows genomic DNA, which was used as a negative control for type I interleukin-1 receptor.



**Figure 3.** Mean ( $\pm$ SE) Cortisol Secretion in Response to Corticotropin and Interleukin-1 $\beta$  in Cultures of Adrenal Adenoma Cells from the Case Patient and Two Other Patients and Normal Adrenal Cells.

Panel A contrasts the dose-dependent effect of interleukin-1 $\beta$  on cortisol production by adrenal adenoma cells from the patient expressing type I interleukin-1 receptors with that of corticotropin. Panel B shows the time-dependent effect of interleukin-1 $\beta$  and corticotropin on cortisol production by adrenal adenoma cells from the patient. Panel C shows the effects of interleukin-1 $\beta$  and corticotropin on adrenal adenoma cells from the patient, adrenal adenoma cells that did not express type I interleukin-1 receptor from two other patients, and normal adrenal cells from two patients. Values are means ( $\pm$ SE) of four incubation mixtures. P values are for the comparison with basal cortisol secretion.

adenomas. No PCR signals were obtained with these primers when either genomic DNA was used or RNA isolated from the patient's adenoma but not treated with reverse transcriptase was used (data not shown).

The clonality assay demonstrated that like other adrenal tumors,<sup>14,15</sup> the patient's adenoma was monoclonal (data not shown).

#### Hormonal Measurements

Corticotropin did not stimulate the secretion of cortisol in cultures of adenoma cells from the patient, whereas interleukin-1 $\beta$  caused a dose-dependent increase in cortisol secretion ( $P<0.001$ ) (Fig. 3A) that was also time dependent (Fig. 3B). The combination of 10<sup>-9</sup> M corticotropin and 10<sup>-8</sup> M interleukin-1 $\beta$  stimulated cortisol production ( $P<0.001$  for the comparison with basal secretion) no more than did 10<sup>-8</sup> M interleukin-1 $\beta$  alone ( $P<0.001$ ) (Fig. 3A).

In contrast, cultured normal adrenal cells responded more strongly to corticotropin ( $P<0.001$  for the comparison with basal secretion) than to interleukin-1 $\beta$  ( $P=0.03$  for the comparison with basal secretion) (Fig. 3C). Similarly, cells from two other cortisol-secreting adenomas responded weakly to interleukin-1 $\beta$  (Fig. 3C).

#### DISCUSSION

This patient's corticotropin-independent adrenal adenoma attracted our attention because of its massive infiltration with CD45- and CD68-containing leukocytes and macrophages, which are a major source of cytokines such as interleukin-1. In contrast to normal adrenal tissue and other adrenal adenomas or carcinomas, this adenoma expressed type I interleukin-1 receptors, and the cells of this tumor secreted cortisol in response to interleukin-1 $\beta$ . In the normal adrenal tissue, interleukin-1 induced a slight increase in cortisol synthesis that was independent of corticotropin. Since interleukin-1 receptors were not detectable on normal adrenal cells, it is possible that

this cytokine activates macrophages, fibroblasts, or adrenomedullary cells to secrete prostaglandins, corticotropin-releasing hormone, or other factors that then stimulate the synthesis and secretion of cortisol by adrenocortical cells.<sup>16</sup>

Infiltration of adrenal tissue by mononuclear cells has been noted in histologic sections from up to 15 percent of patients with Cushing's syndrome due to diffuse and nodular adrenal hyperplasia.<sup>17,18</sup> A functional relation of these cells to adrenocortical hypersecretion was not suspected or studied, however.

In animals, interleukin-1 $\beta$  induces the proliferation and regeneration of adrenal tissue.<sup>19,20</sup> Yet we did not find aberrant expression of interleukin-1 receptors on normal adrenal cells or cells from other cortisol-producing adrenal tumors. Therefore, it is unlikely that the expression of interleukin-1 receptors by our patient's tumor was merely the consequence of unrestrained tumor growth.

High local concentrations of interleukin-1 combined with aberrant expression of type I interleukin-1 receptors by a particular population of adrenocortical cells, followed by clonal expansion of the latter, may have provided the basis for tumor formation in this patient. The tumor cells were not responsive to corticotropin, and the strong expression of interleukin-1 receptors is consistent with the marked response to interleukin-1, leading to hypersecretion of cortisol. This case, in which aberrant expression of a cytokine receptor appears to be responsible for hormone secretion, expands the recent concept that corticotropin-independent adrenal Cushing's syndrome may be due to aberrant adrenocortical expression of receptors for hormones and neuropeptides.

Supported by grants from the Mildred Scheel Stiftung (10-1070-Re I; to Dr. Bornstein), Boehringer Ingelheim (to Dr. Willenberg), and the Deutsche Forschungsgemeinschaft (EH 161/1-1; to Dr. Ehrhart-Bornstein) and by a Heisenberg grant (to Dr. Bornstein).

## REFERENCES

1. Reincke M, Mora P, Beuschlein F, Arlt W, Chrousos GP, Allolio B. Deletion of the adrenocorticotropic receptor gene in human adrenocortical tumors: implications for tumorigenesis. *J Clin Endocrinol Metab* 1997;82:3054-8.
2. Lacroix A, Bolté E, Tremblay J, et al. Gastric inhibitory polypeptide-dependent cortisol hypersecretion — a new cause of Cushing's syndrome. *N Engl J Med* 1992;327:974-80.
3. Reznik Y, Allali-Zerah V, Chayvialle JA, et al. Food-dependent Cushing's syndrome mediated by aberrant adrenal sensitivity to gastric inhibitory polypeptide. *N Engl J Med* 1992;327:981-6.
4. Lacroix A, Tremblay J, Touyz RM, et al. Abnormal adrenal and vascular responses to vasopressin mediated by a V1-vasopressin receptor in a patient with adrenocorticotropic-independent macronodular adrenal hyperplasia, Cushing's syndrome, and orthostatic hypotension. *J Clin Endocrinol Metab* 1997;82:2414-22.
5. Lacroix A, Tremblay J, Rousseau G, Bouvier M, Hamet P. Propranolol therapy for ectopic  $\beta$ -adrenergic receptors in adrenal Cushing's syndrome. *N Engl J Med* 1997;337:1429-34.
6. Sims JE, Gayle MA, Slack JL, et al. Interleukin 1 signaling occurs exclusively via the type I receptor. *Proc Natl Acad Sci U S A* 1993;90:6155-9.
7. Sims JE, Giri JG, Dower SK. The two interleukin-1 receptors play different roles in IL-1 actions. *Clin Immunol Immunopathol* 1994;72:9-14.
8. Bornstein SR, González-Hernández JA, Ehrhart-Bornstein M, Adler G, Scherbaum WA. Intimate contact of chromaffin and cortical cells within the human adrenal gland forms the cellular basis for important intraadrenal interactions. *J Clin Endocrinol Metab* 1994;78:225-32.
9. Tada M, Diserens AC, Desbaillets I, Jaufeerally R, Hamou MF, de Tribolet N. Production of interleukin-1 receptor antagonist by human glioblastoma cells in vitro and in vivo. *J Neuroimmunol* 1994;50:187-94.
10. Stratakis CA, Jenkins RB, Pras E, et al. Cytogenetic and microsatellite alterations in tumors from patients with the syndrome of myxomas, spotty skin pigmentation, and endocrine overactivity (Carney complex). *J Clin Endocrinol Metab* 1996;81:3607-14.
11. Allen RC, Zoghbi HY, Moseley AB, Rosenblatt HM, Belmont JW. Methylation of HpaII and HhaI sites near the polymorphic CAG repeat in the human androgen-receptor gene correlates with X chromosome inactivation. *Am J Hum Genet* 1992;51:1229-39.
12. Páth G, Bornstein SR, Ehrhart-Bornstein M, Scherbaum WA. Interleukin-6 and the interleukin-6 receptor in the human adrenal gland: expression and effects on steroidogenesis. *J Clin Endocrinol Metab* 1997;82:2343-9.
13. Glasow A, Bredert M, Haidan A, Anderegg U, Kelly PA, Bornstein SR. Functional aspects of the effect of prolactin (PRL) on adrenal steroidogenesis and distribution of the PRL receptor in the human adrenal gland. *J Clin Endocrinol Metab* 1996;81:3103-11.
14. Beuschlein F, Reincke M, Karl M, et al. Clonal composition of human adrenocortical neoplasms. *Cancer Res* 1994;54:4927-32.
15. Gicquel C, Leblond-Francillard M, Bertagna X, et al. Clonal analysis of human adrenocortical carcinomas and secreting adenomas. *Clin Endocrinol (Oxf)* 1994;40:465-77.
16. Ehrhart-Bornstein M, Hinson JP, Bornstein SR, Scherbaum WA, Vinson GP. Intra-adrenal interactions in the regulation of adrenocortical steroidogenesis. *Endocr Rev* 1998;19:101-43.
17. Wegienka LC, Komarny LE, Wuopper KD, Forsham PH. Cushing's syndrome with adrenal medullary insufficiency and adrenal autoantibodies. *Lancet* 1966;1:741-3.
18. Andrada JA, Murray FT, Andrada EC, Ezrin C. Cushing's syndrome and autoimmunity. *Arch Pathol Lab Med* 1979;103:244-6.
19. Hanley N, Williams BC, Nicol M, Bird IM, Walker SW. Interleukin-1 beta stimulates growth of adrenocortical cells in primary culture. *J Mol Endocrinol* 1992;8:131-6.
20. Zieleniewski W, Zieleniewski J, Stepień H. Effect of interleukin-1 $\alpha$ , IL-1 $\beta$  and IL-1 receptor antibody on the proliferation and steroidogenesis of regenerating rat adrenal cortex. *Exp Clin Endocrinol Diabetes* 1995;103:373-7.