Biosynthesis of Vasopressin In Vitro and Ultrastructure of a Bronchogenic Carcinoma

PATIENT WITH THE SYNDROME OF INAPPROPRIATE SECRETION OF ANTIDIURETIC HORMONE

JACK M. GEORGE, CHARLES C. CAPEN, and AUDRA S. PHILLIPS

From the Division of Endocrinology, Department of Medicine, and Department of Veterinary Pathology, Ohio State University, Columbus, Ohio 43210

ABSTRACT Tumors from patients with the syndrome of inappropriate secretion of antidiuretic hormone (SIADH) have been found to contain large amounts of the antidiuretic hormone vasopressin. A lung tumor from a patient with hyponatremia most likely due to SIADH was removed at surgery and found to contain 23.5 mU vasopressin/g wet weight by radioimmunoassay Slices of this tumor were incubated with phenylalanine-^{3}H. Arginine vasopressin-^{3}H was purified from the incubate by Sephadex G-25 column chromatography in two different systems, performic acid oxidation, and gradient elution column chromatography with diethylaminoethyl Sephadex. As oxidation of vasopressin results in drastic conformational change with breaking of the ring of the cyclic polypeptide and addition of two cysteic acid residues per molecule, the radioactive material which eluted coincident with vasopressin both before and after this procedure was considered to be arginine vasopressin-^{3}H. To our knowledge this is the first demonstration of in vitro biosynthesis of vasopressin by a tumor from a patient with SIADH.

Ultrastructurally, the bronchogenic carcinoma was composed of small undifferentiated and granulated cells. The granulated neoplastic cells had well developed organelles (endoplasmic reticulum, free ribosomes) concerned with protein synthesis. Secretion granules present in the tumor cells were small, surrounded by a limiting membrane, and resembled those reported in polypeptide hormone-secreting cells.

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INTRODUCTION

Since delineation of the syndrome of inappropriate secretion of antidiuretic hormone in association with malignant neoplasms (1) an increasing amount of circumstantial evidence has indicated that the hormone involved is the naturally occurring antidiuretic hormone in man, arginine vasopressin (AVP)[1] and that it is produced by the neoplasm. This syndrome is one of a number of ectopic hormone syndromes and it now appears that malignant neoplasms have the capacity to synthesize and release one or more of most of the known polypeptide hormones. However, in no case has any ectopic hormone been isolated in pure form and its amino acid sequence determined to prove that it is identical with the natural hormone. Furthermore, it has not been shown that a neoplasm removed from a patient with SIADH is capable in virto of synthesizing AVP.

This report concerns a patient with undifferentiated carcinoma of the lung who presented with hyponatremia and additional clinical evidence that inappropriate secretion of vasopressin was present. A sample of the carcinoma removed at surgery was shown by radioimmunoassay to contain amounts of vasopressin far greater than the plasma concentration of 15 μU/ml or less which has been found in patients with this syndrome (2). Another sample of tumor was incubated in vitro and incorporation of phenylalanine-^{3}H into vasopressin was demonstrated.

CASE REPORT

History. U. H. 281-01-8165 C. C. a 68 yr old male retired machinist entered University Hospital because of

[1] *Abbreviations used in this paper:* AVP, arginine vasopressin; SIADH, syndrome of inappropriate secretion of antidiuretic hormone.

an abnormal chest X-ray. He had been in excellent health until 2 yr before admission when he noted melena and a peptic ulcer was diagnosed. The melena ceased after medical treatment for the peptic ulcer but 6 wk before his admission to University Hosiptal he was hospitalized elsewhere because of hematemesis and a peptic ulcer was again seen on X-ray. In the 2 months before that admission, he had noted a 20 lb. weight loss with persistent cough, increased sputum production, and intermittent hemoptysis. Opacification in the upper lobe of the left lung was seen on chest X-ray and the patient was subsequently referred to University Hospital. He had smoked one to two packs of cigarettes per day for 30 yr.

Physical examination. Blood pressure, pulse, and respiration rate were normal. There was marked clubbing of the fingers. The optic fundi showed some arteriolar narrowing. Bronchial breath sounds were heard over the left upper posterior lung field. The liver was enlarged to 7 cm below the right costal margin. The stool was negative for occult blood.

Laboratory studies. The hematocrit was 43% with 12,200 total leukocytes per mm^3. Urinalysis was normal and urine specific gravity was 1.015. Prothrombin, albumin, globulin, alkaline phosphatase, calcium, amylase, glutamic oxalacetic transaminase, and lactic dehydrogenase in the serum were all normal. Electrocardiogram was normal except for nonspecific ST-T wave changes. Concentrated sputum smear for acid fast organisms was negative, but Papanicolaou's smear of sputum showed squamous cells suspicious of a malignant neoplasm. Moderate obstructive impairment was found by spirometry. On chest X-ray, collapse of the anterior segment of the left upper lobe was seen due to a probable mass. Bronchograms confirmed the occlusion of the bronchus of the anterior segment of the left upper lobe.

On admission, the serum sodium was 119 mEq/liter, chloride 84 mEq/liter, potassium 4.3 mEq/liter, bicarbonate 25 mEq/liter, blood urea nitrogen 12 mg/100 ml, creatinine 0.9 mg/100 ml, uric acid 1.8 mg/100 ml. He had an abnormal 2 hr postprandial serum glucose of 160 mg/100 ml. The hyponatremia persisted during the 5 preoperative days with serum sodium ranging between 118 and 123 mEq/liter and during this time urine specific gravity was 1.015 and 24 hr urine excretion of sodium was 110 mEq/24 hr on one occasion. On the 6th hospital day a thoracotomy was performed and a lung tumor 5 cm in diameter was found in the apical posterior segment of the left lung. A pneumonectomy was performed and on microscopic examination the tumor was seen to be an undifferentiated carcinoma. 6 of the 17 hilar lymph nodes removed contained carcinoma. His postoperative course was complicated by development of a bronchopleural fistula which was treated by insertion of a chest tube but his general condition gradually deteriorated and he died on the 17th postoperative day. Permission for autopsy was not granted. During the postoperative period his hyponatremia was corrected by infusion of sodium chloride.

IMMUNOASSAY

Arginine vasopressin was purified from bovine posterior pituitary glands (3), coupled to egg albumin with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl (4), and injected subcutaneously into rabbits with complete Freund's adjuvant. After three biweekly injections, periodic booster injections were given followed 7–10 days later by bleeding. The antiserum thus collected was tested for its ability to bind AVP-^{125}I and the antiserum with greatest avidity was used for radioimmunoassay. AVP was labeled with ^{125}I by a modification (5) of the method of Hunter and Greenwood (6).

Radioimmunoassay was performed in 1 ml of 0.5 M potassium phosphate buffer pH 8.1 containing 2% nonimmune rabbit serum, 0.01 M ethylenediaminetetraacetate, antiserum in 1:30,000 dilution, approximately 30 pg AVP-^{125}I, goat anti-rabbit gamma globulin in 1:10 dilution and differing amounts of unlabeled AVP or the biologic sample to be assayed. After these ingredients were mixed, the tubes were allowed to stand overnight at 4°C and the AVP-^{125}I bound to antibody was collected as a precipitate by centrifugation. The per cent of AVP-^{125}I bound to antibody was graphed as a function of the log of unlabeled AVP present and a standard curve (Fig. 1) obtained. There was no significant displacement of bound AVP-^{125}I by 10,000 pg oxytocin.

Lung tumor tissue which had been frozen immediately at the time of surgery was extracted in 5.5 N formic acid (pH 1.1), and the precipitated protein collected by centrifugation and discarded. The extract was neutralized with ammonium hydroxide and assayed for AVP. Each gram wet weight of tumor was found to contain 58.7 ng or 23.5 mU (assuming 400 U/mg). Assay of 5 and 10-fold dilutions of tumor extract fell along the standard curve for authentic AVP (Fig. 1).

INCUBATION AND ISOLATION OF AVP FROM TUMOR

At the time of surgery 2.8 g of lung tumor was immediately cut in 0.5 mm slices using a Stadie-Riggs microtome and placed in a flask in 20 ml of Krebs-Ringer bicarbonate-phosphate buffer pH 7.4 containing freshly dialyzed bovine serum albumin 40 mg/100 ml, glucose 230 mg/100 ml, and 0.8 mCi of phenylalanine-^{3}H (2.8 Ci/mmole) which we had demonstrated to be chromatographically pure. The flask was incubated at 37°C in 95% O_2 + 5% CO_2 for 5 hr in a metabolic shaker. At the end of the incubation, the tissue plus medium

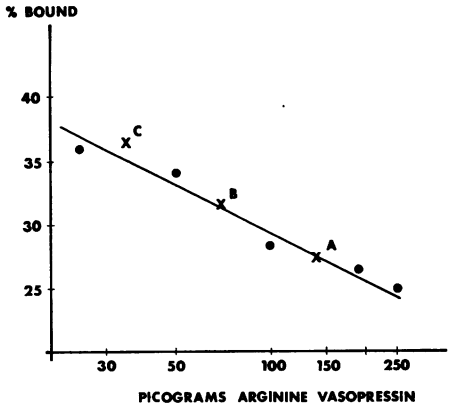

FIGURE 1 Radioimmunoassay of lung tumor extract. The amount of arginine vasopressin-^{125}I bound by antiserum is expressed as a function of picograms arginine vasopressin present. A, B, C are dilutions of tumor extract, 1 : 10, 1 : 50, 1 : 100, respectively.

were homogenized together using a teflon pestle homogenizer and the homogenate was centrifuged for 225,000 $g \times$ min. The pellet was discarded and the supernatant lyophilized.

The lyophilized powder was dissolved in 0.1 M ammonium acetate buffer pH 4.5 and chromatographed on a 2 × 150 cm column of Sephadex G-25 in the same buffer. A peak of radioactivity corresponding to the chromatographic mobility of authentic AVP was collected, lyophilized, and rechromatographed with 3 mg added unlabeled AVP on a 2 × 150 cm column of Sephadex G-25 in 0.1 M formic acid. A small peak of radioactivity emerged at the void volume while the major peak, comprising 98% of the total radioactivity in the two peaks, was detected at 2.4 void volumes. This was coincident with the ultraviolet absorbing peak of added AVP and was collected, lyophilized, oxidized with performic acid to form the dicysteic acid derivative of vasopressin (7). The oxidized sample was applied to a 1.5 × 20 cm column of DEAE Sephadex and eluted using a linear gradient from 0.002 M ammonium acetate pH 7.4 to 0.3 N hydrochloric acid. A majority of the radioactivity (87%) emerged from the column shortly after the beginning of the gradient coincident with the mobility of phenylalanine but there was a peak of 3,000,000 cpm that eluted with the UV absorbing dicysteic acid derivative of vasopressin. Another peak of UV absorbing material eluted from the column in the early phase of the gradient and was shown by independent experiments to most likely be a product of the performic acid used in the oxidation as has been previously suggested (7). The purified arginine vasopressin-^{3}H was not tested for immunoreactivity because performic acid oxidized AVP does not react with our antiserum.

Because of the known affinity of amino acids of high specific activity for polypeptides (8) we were concerned that phenylalanine-^{3}H had been carried through the isolation procedure associated with vasopressin. This concern was supported by an experiment in which phenylalanine-^{3}H was mixed with unlabeled AVP, oxidized with performic acid, and chromatographed on DEAE Sephadex with the gradient elution described above. Although the majority of the phenylalanine-^{3}H eluted early, significant radioactivity eluted later with UV absorbing oxidized AVP. This experiment was then repeated with the addition of sodium dodecyl sulfate to the eluting buffer such that a concentration of 0.1% was maintained during development of the pH and ionic strength gradients and this time negligible phenylalanine-^{3}H eluted with oxidized AVP. With this information in hand the radioactive peak from the tumor incubation which had eluted with oxidized arginine vasopressin during gradient elution from DEAE Sephadex was rechromatographed in the presence of 0.1% sodium dodecyl sulfate and 92% of the radioactivity remained with the oxidized AVP peak as evidence that the lung tumor had incorporated phenylalanine-^{3}H into arginine vasopressin-^{3}H (Fig. 2).

MATERIALS AND METHODS FOR ELECTRON MICROSCOPY

Multiple blocks of tissue from the primary lesion in the lung were prepared for study by electron microscopy. The tissue was cut under fixative into 0.5–1.0 mm cubes immediately after surgical excision. The tissue was fixed in 3% glutaraldehyde with postfixation in 1% osmium tetroxide with added calcium chloride. The blocks were dehydrated through ascending concentrations of ethyl alcohol, trans-

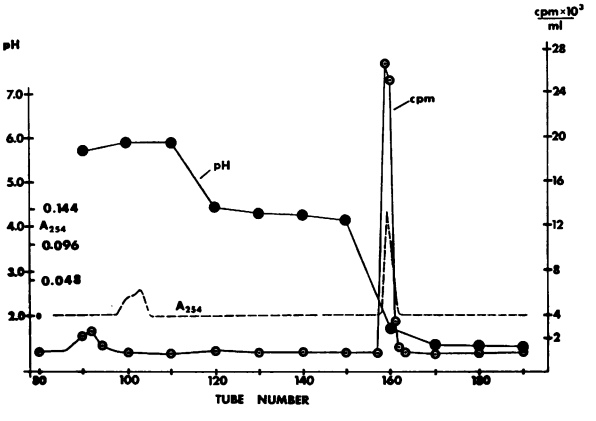

FIGURE 2 Final column chromatography of tumor incubate. After incubation of lung tumor slices with phenylalanine-^{3}H, the incubate was purified by multiple column chromatography and then oxidized along with purified arginine vasopressin. The 1.5 × 20 cm column of diethylaminoethyl Sephadex was eluted with 0.1% sodium dodecyl sulfate and a stepwise pH gradient. The ultraviolet absorbing peak that eluted with the major peak of radioactivity is due to the dicysteic acid derivative of the added arginine vasopressin.

ferred to propylene oxide, and embedded in Maraglas. Sections were cut at 500 A with diamond knives on a Reichert OmU2 ultramicrotome (American Optical Corp., Buffalo, N. Y.) and mounted on 200- and 400-mesh copper grids. The sections were stained with uranyl acetate and lead hydroxide, and evaluated with a Philips 200 electron microscope (Philips Electronic Instruments, Mount Vernon, N. Y.) The findings in this tumor were compared with tissue from four patients with bronchogenic carcinoma who did not have clinical evidence of endocrine activity. The magnification listed for the electron micrographs represents the actual magnification after printing.

ELECTRON MICROSCOPY

The predominant type of cell present in the pulmonary carcinoma from the patient with the syndrome of inappropriate antidiuretic hormone was relatively small, pleomorphic, and undifferentiated. A large, often irregularly shaped, nucleus occupied a major portion of the cell (Fig. 3). There was peripheral clumping of nuclear chromatin and the nucleolus was prominent.

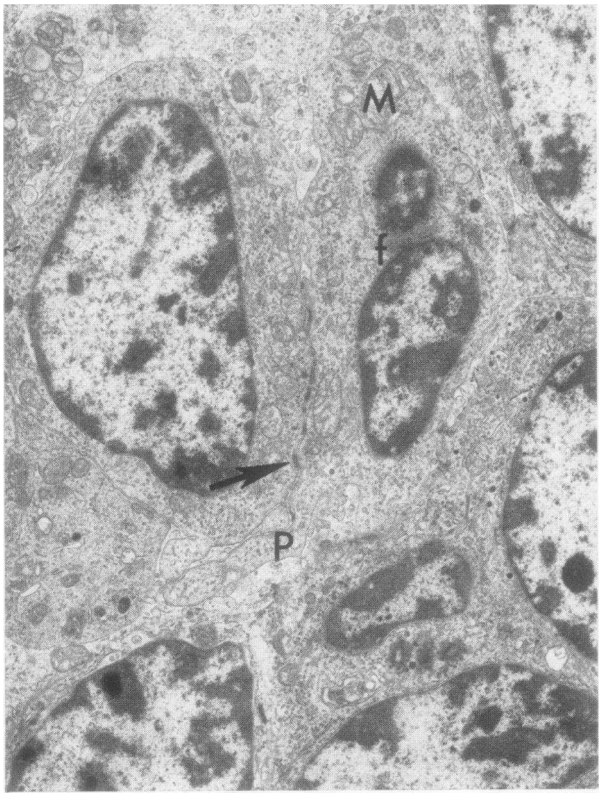

FIGURE 3 Electron micrograph illustrating the predominant type of cell in the undifferentiated carcinoma. The nucleus occupies a large portion of the cell area and nuclear chromatin is clumped peripherally. Plasma membranes of adjacent neoplastic cells are attached by multiple desmosomes (arrow) and long cytoplasmic projections (P) extend into the intercellular spaces. The cytoplasm contains many aggregations of free ribosomes, large mitochondria (M), and microfilaments (f). × 8200.

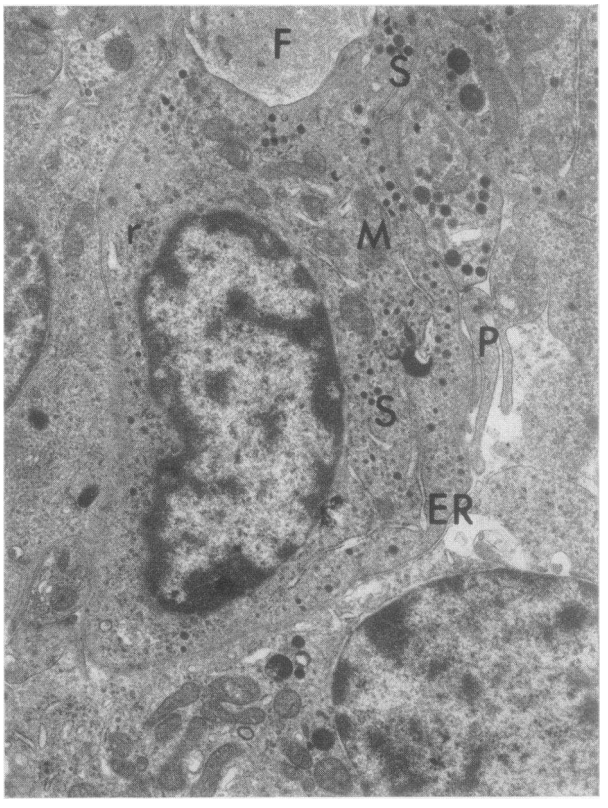

FIGURE 4 Neoplastic cell with numerous intracytoplasmic secretory granules (S) and long profiles of endoplasmic reticulum with attached ribosomes. These cells were observed less frequently than the poorly differentiated neoplastic cells (at left and bottom). In addition to secretion granules, large mitochondria (M) and aggregations of free ribosomes (r) were present in the cytoplasm. Cytoplasmic projections (P) extended into the prominent intercellular spaces which often contained a network of intermeshed fibrils (F). × 11,300.

Plasma membranes of adjacent neoplastic cells were often indistinct and were joined by multiple desmosomes and uncomplicated interdigitations. Projections of cytoplasm frequently extended as pseudopods between adjacent cells (Fig. 3). An interwoven network of fine fibrils was present in the intercellular spaces between certain neoplastic cells (Fig. 4). The principal cytoplasmic organelles were free ribosomes aggregated into rosettes and large mitochondria. Membranes of the rough endoplasmic reticulum and Golgi apparatus were poorly developed in most neoplastic cells of this type. Lipid bodies, microfilaments, small groups of glycogen particles, and lipofuscin granules were observed in occasional neoplastic cells.

Granulated neoplastic cells were observed less frequently and usually in small groups interspersed between the anaplastic cells. They had a more abundant cytoplasmic area containing numerous electron-dense

secretory granules and well-developed organelles concerned with protein synthesis and packaging of secretory products (Figs. 4 and 5). Long profiles of rough endoplasmic reticulum, occasionally aggregated into lamellar arrays, were present in the cytoplasm in addition to rosettes of free ribosomes. A prominent Golgi apparatus associated with vacuoles and small granules was often located near the nucleus. There was considerable variation in morphology of the many large mitochondria present in the neoplastic cells (Fig. 6). They were either tubular in profile, dumbbell-shaped, or circular (cup-shaped) with plate-like and tubular cristae. Mitochondrial peculiarities have been reported previously by other investigators in different types of bronchogenic carcinomas (9). Lipofuscin granules and lysosome-like dense bodies were observed in certain granulated neoplastic cells.

The secretion granules were extremely electron-dense and surrounded by a continuous limiting membrane (Fig. 5). They ranged in size from 130 to 240 mμ and

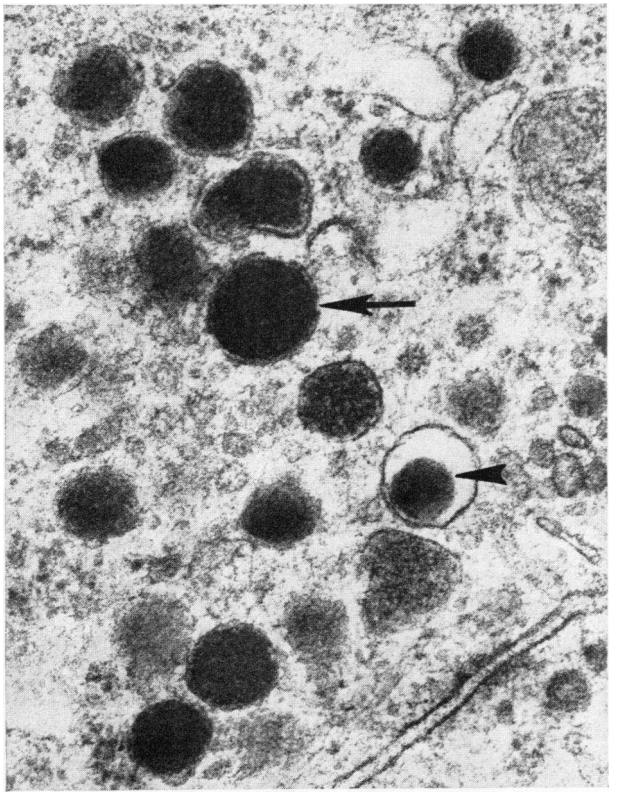

FIGURE 5 Electron micrograph illustrating at higher magnification the small secretory granules observed in certain neoplastic cells. They were extremely electron-dense and surrounded by a limiting membrane. The submembranous space was usually narrow (long arrow) but was expanded (short arrow) around the internal core of an occasional secretion granule. Plasma membrane of cell is at lower right. × 16,900.

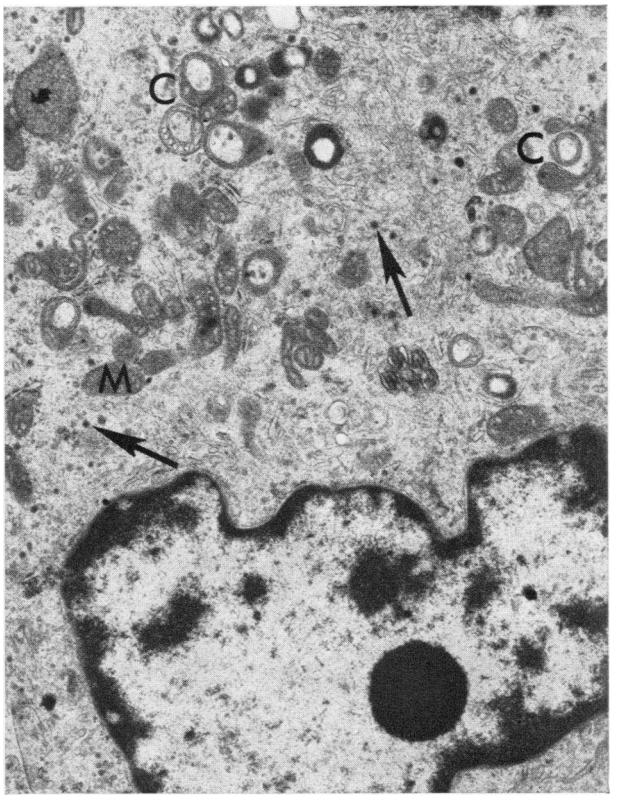

FIGURE 6 Neoplastic cell with a large cytoplasmic area illustrating the striking variation in mitochondrial structure and scattered small secretory granules (arrows). The mitochondria were either tubular (M), dumbbell-shaped, or circular (C, cup-shaped). The nucleolus was prominent and the nuclear chromatin was clumped peripherally. × 15,300.

were composed of small dense particles. The submembranous space separating the limiting membrane from the internal core of the granule was usually narrow but was expanded considerably in an occasional granule. Granulated neoplastic cells of this type were observed in the four pulmonary carcinomas from patients without clinical evidence of endocrine activity but much less frequently than in the tumor of the patient reported herein.

DISCUSSION

The available clinical data from this patient, although far from complete, suggest the presence of the syndrome of inappropriate vasopressin secretion as delineated by Schwartz and Bartter (1). Persistent hyponatremia was present without clinical hypovolemia and the urine was shown to contain significant amounts of sodium and to be concentrated when the serum was markedly dilute. The normal serum creatinine and blood urea nitrogen suggest normal renal function and there was no clinical evidence of adrenal insufficiency. After removal of the

lung tumor, the serum sodium could be restored to normal with intravenous sodium chloride solution. This maneuver is notoriously difficult to accomplish in the presence of the syndrome. The diagnosis of inappropriate vasopressin secretion in this patient was supported by finding 23 mU vasopressin/g wet weight in the lung tumor.

The demonstration of vasopressin by bioassay in tumors from patients with this syndrome has been increasingly frequent since Amatruda, Mulrow, Gallagher, and Sawyer demonstrated antidiuretic activity in such a tumor, found it to be inactivated by thioglycolate which reduces the disulfide bridge, and thus breaks the ring of vasopressin and showed that such material is not found in similar tumors from patients without the clinical syndrome (10). In 1964 Bower, Mason, and Forsham (11) demonstrated antidiuretic activity in plasma as well as lung tumor and metastases from such a patient, again using the rat bioassay. They also performed bioassays in rats with stereotactically placed hypothalamic lesions and permanent diabetes insipidus and proved that the active principle of the tumor was not acting in the assay animal by stimulating the hypothalamus or posterior pituitary gland to release vasopressin. At autopsy, their patient was found to have complete destruction of the posterior pituitary gland by metastasis and absence of aldehyde fuchsin staining material in the hypothalamus. The hypothalamic and posterior pituitary material that takes up this special stain has been shown to behave as the neurosecretory material in a variety of physiological situations and is presumed to represent vasopressin, oxytocin, and the neurophysins, proteins that are synthesized and transported with these hormones. The lack of such material is good evidence that the excess vasopressin secretion in this patient was truly ectopic and was not being secreted by the pituitary. The most complete pharmacologic characterization of ectopic antidiuretic activity was reported in 1967 by Sawyer (12). He found that the tumor extract was similar to AVP in the following properties: soluble in trichloroacetic acid, absorbed by IRC-50 ion exchange resin, ratio of antidiuretic to vasopressor to toad bladder activity (1:1:1), reversibly bound by neurophysin, inactivated by thioglycolate, pregnancy plasma, trypsin and chymotrypsin, but not inactivated by normal plasma or by pepsin. Lipscomb, Wilson, Retiene, Matsen, and Ward in 1968 reported that ectopic antidiuretic activity eluted from Sephadex G-25 at the same volume as AVP and that both traveled together through a countercurrent distribution (13). In addition to bioassay, vasopressin has also been detected by radioimmunoassay in tumors of patients with the syndrome of inappropriate antidiuretic hormone secretion (2, 14, 15). In 1966 Utiger found that the extract from a tumor of a patient with this syndrome displaced vasopressin from a specific antiserum to vasopressin and on column chromatography using Sephadex G-50, the immunoreactivity was considerably retarded after the protein peak as would be expected of a small polypeptide (14). Our patient also had radioimmunoassayable vasopressin in his lung tumor. When the tumor extract was diluted over a 10-fold range, the dilutions fell along the standard curve (Fig. 1) suggesting an immunologic resemblance between tumor principle and natural vasopressin.

The evidence cited above strongly suggests that some malignant neoplasms synthesize AVP. We have sought to prove this by demonstrating in vitro synthesis of radiolabeled vasopressin from radiolabeled amino acid in such a tumor. The chromatographic behavior of a tritium-labeled substance from the tumor and particularly that of its performic acid-oxidized derivative, which behaved as the dicysteic acid derivative of AVP, give increasing confidence that arginine vasopressin-^{3}H was formed by the tumor. However, radiolabeled amino acids of high specific activity are notoriously difficult to separate from unlabeled polypeptides by conventional methods. The demonstration that such association could occur during column chromatography but that it could be prevented by the addition of 0.1% sodium dodecyl sulfate (a detergent) tends to exclude such nonspecific association as an explanation of our findings. There can be no conclusive proof of radiochemical purity short of isolation and proof of structure of the radiolabeled molecule which is not practicable when dealing with such minute quantities. The proof in this experiment rests on the assumption that even if other small radiolabeled polypeptides synthesized by the tumor were eluted with AVP from Sephadex G-25 in two different systems, it is highly unlikely that when oxidized with performic acid these oxidized contaminants would be similar enough in charge and conformation to elute with oxidized AVP. The conformational change of vasopressin resulting from performic acid oxidation is drastic and involves conversion of a cyclic to a linear polypeptide with two cysteic acid groups added per molecule.

The 3,000,000 cpm of arginine vasopressin-^{3}H purified from the incubation medium represents approximately 0.8% incorporation of the 0.8 mCi of phenylalanine-^{3}H incubated with the tumor. This corresponds to a synthetic rate of 170 pmoles AVP-hr per g wet weight of tumor. As the extensive purification must have resulted in large losses of arginine vasopressin-^{3}H and the efficiency of incorporation of labeled by the tissue is unknown the true rate of synthesis must be far greater. A rate of AVP synthesis of about 1 pmole/hr per guinea pig hypothalamus has been found in in vitro incubation (16). There is no comparable data available for human hypothalamus.

Ultrastructural evaluation of the bronchogenic carcinoma associated with the syndrome of inappropriate antidiuretic hormone secretion supported the in vitro evidence of synthesis of vasopressin by the tumor cells. The granulated neoplastic cells had well developed organelles concerned with protein synthesis and packaging of secretory products. They shared many similarities ultrastructurally with normal polypeptide hormone-synthesizing endocrine cells (17–20), including neurosecretory neurones in the supraoptic nucleus that produce antidiuretic hormone (21). The hormonal activity of several endocrine cells has been shown to be associated with the secretory granule fraction (22, 23). The secretion granules were surrounded by a continuous limiting membrane and were in a similar size range as neurosecretory granules in the hypothalamus (21).

The finding of granulated cells in the bronchogenic carcinoma differed from the results of the only other reported case in which a lung tumor associated with the syndrome of inappropriate antidiuretic hormone secretion was examined by electron microscopy. Whitelaw (24) evaluated an oat-cell carcinoma of the lung from a patient with this syndrome but detected no cellular organization suggesting hormone production. However, other investigators have reported that oat-cell carcinomas and bronchial carcinoids of the lung with endocrine activity (25), as well as thymic neoplasma secreting adrenocorticotrophic hormone (26), are composed of neoplastic cells containing secretion granules and lamellar arrays of endoplasmic reticulum similar to that seen in our patient.

The predominant undifferentiated cells in the bronchogenic carcinoma were small and pleomorphic with large nuclei and a narrow rim of cytoplasm. Secretion granules were not present and cytoplasmic organelles, other than mitochondria and free ribosomes, were poorly developed. These anaplastic tumor cells shared many ultrastructural characteristics with the small ("oat") cell type of bronchogenic carcinoma that has been reported previously (27–29). They could be readily differentiated from squamous cell carcinomas of the lung by the lack of tonofilaments and keratohyaline glanules, and from pulmonary adenocarcinomas by the absence of acinar formation with microvilli and large secretory vacuoles (28). In addition, the poorly differentiated tumor cells lacked the unique multilamellar bodies and cytoplasmic inclusions with flocculent material characteristic of granular pneumocytes (alveolar type II) which comprise alveolar cell carcinomas (30).

To our knowledge, this is the first demonstration in vitro of synthesis of vasopressin by a tumor from a patient with the syndrome of inappropriate antidiuretic hormone secretion. The question remains why some malignant neoplasms, predominantly lung tumors, synthesize vasopressin or other polypeptide hormones while others do not. That all cells in the body have the genetic capacity to perform any function of the body is supported by an experiment in which the nucleus of an intestinal epithelial cell of an adult frog was transplanted into an enucleated frog ovum and a normal adult frog resulted (31).

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