6. The neutrophil preparations contained fewer than one platelet per ten neutrophils, but we cannot rule out the platelet as a potential source of chlorinatable amines. However, neutrophils prepared from defibrinated blood (fewer than one platelet per 20 neutrophils) generated identical quantities of the *N*-chloroamine.
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Specific Endocrine Tissue Marker Defined by a Monoclonal Antibody

Abstract. *One of two mouse monoclonal antibodies (LK2H10) produced by hybridoma technology against a human endocrine tumor (pheochromocytoma) demonstrated specific immunoreactivity for 69 normal and neoplastic endocrine cells and tissues known to contain secretory granules. This immunoreactivity was specific, since other normal tissues, tumors from endocrine cells without granules, and tumors from other nonendocrine tissues were negative when tested with antibody LK2H10. The antibody reacted with human fetal adrenal medulla and human pancreatic endocrine cells and with adrenal medullary cells from monkeys and pigs. The antigen detected by antibody LK2H10 is associated with cytoplasmic secretory granules, has an estimated molecular weight of 68,000, and may be related to human chromogranin.*

Immunohistochemical techniques in which polyclonal antisera are used to identify various peptide hormones and amines have been indispensable in characterizing cells and tissues of the amine precursor uptake and decarboxylation (APUD), or diffuse neuroendocrine (DNE), system and tumors derived from these tissues (*1*). Conventional techniques used to identify neurosecretory granules in these cells, such as electron microscopy and the various silver stains (*2*), are relatively nonspecific compared to immunohistochemical methods with polyclonal antisera. With hybridoma technology (*3*), one may now generate virtually unlimited supplies of monoclonal antibodies to previously undetected hormones and proteins associated with the complex secretory granules in cells of the DNE system, which may help to further characterize this complex system of cells. Thus, monoclonal antibodies produced against purified synaptic junctions (*4*) or neural plasma membranes (*5*) have been found to cross-react extensively with cells of the DNE system. In addition, reovirus-induced polyendocrinopathy of SVL/J mice enabled the isolation of a variety of mouse monoclonal autoantibodies reactive with a number of polypeptide endocrine hormones (*6*). In this report we describe a monoclonal antibody that reacts with a 68,000-dalton protein associated with endocrine secretory granules. The availability of such a specific monoclonal antibody will allow for a simple and highly reproducible method to visualize cells with neurosecretory granules in endocrine tissues of human and certain vertebrate species.

It was our intention to use the hybridoma technique for developing monoclonal antibodies that would identify new endocrine markers in tissue specimens (Formalin-fixed, paraffin-embedded) routinely prepared and processed by the pathology laboratory. We immunized BALB/c mice with 1-mm^3 pieces of a human pheochromocytoma that had been minced with a razor blade and mixed with Freund's adjuvant before intraperitoneal injection. After five biweekly injections, a mouse was killed and its spleen cells were fused with NS-1 mouse myeloma cells (*7*). After 14 days in medium containing hypoxanthine, aminopterin, and thymidine, culture supernatant fluids from growing clones were screened for antibody activity against cryostat sections of the original tumor by indirect immunofluorescence. After this step, 50 clones were selected and 2 weeks later retested for reactivity with cryostat sections and Formalin-fixed, paraffin-embedded sections of the same tumor by avidin-biotin complex (ABC) (*8*) peroxidase staining (Vector Laboratories).

Table 1. Immunohistochemical reactivity of monoclonal antibody LK2H10 with normal human adult and fetal endocrine tissue. Numbers in parentheses are numbers of cases examined.

Tissue	Reactivity
Adult	
Adrenal medulla (5)	Yes
Adrenal cortex (5)	No
Gastrointestinal-endocrine (8)	Yes
Exocrine pancreas (5)	No
Endocrine pancreas (5)	Yes
Parathyroid (2)	Yes
Anterior pituitary (3)	Yes
Posterior pituitary (5)	No
Thyroid C cells (5)	Yes
Thyroid follicular cells (5)	No
Fetal (13 to 18 weeks)	
Adrenal medulla (4)	Yes
Endocrine pancreas (4)	Yes

Two hybridomas, designated LK2H10 and LK6D10, were selected for additional testing when their antibodies were found to react with tumor cells, but not connective tissue, in cryostat and Formalin-paraffin sections. Although both antibodies reacted exclusively in initial studies with endocrine tissues, only clone LK2H10 was successfully subcloned and thus available for more extensive testing in Formalin-paraffin sections. Reactivity of antibody LK2H10 with endocrine tissues could be detected by ABC peroxidase staining with up to a 100-fold dilution of hybridoma culture fluid. Thus a single large batch of LK2H10 culture fluid was collected and used throughout this study at a tenfold dilution. As a negative control for staining, we used culture fluids containing a monoclonal antibody to an antigenic determinant of human I-region-associated antigens that is denatured in Formalin-paraffin sections. No background staining was observed with hybridoma culture fluids containing our control antibody even when it was tested undiluted by the ABC peroxidase method.

Normal endocrine tissues that were reactive with LK2H10 are listed in Table 1. Immunostaining was done with the ABC peroxidase method (*8*) using a one- to tenfold dilution of the monoclonal antibody for 1 hour at 37°C. The intensity of immunostaining with the normal pancreatic islet was variable. Glucagon-producing cells showed the greatest immunoreactivity, while insulin-producing cells showed the least. Endocrine tumors that showed positive immunoreactivity included pheochromocytomas (five cases), pituitary adenomas (five), medullary thyroid carcinomas (five), pancreatic endocrine neoplasms (five), parathyroid adenomas (two), carcinoids (five), and paragangliomas (two). Endocrine tissues and tumors that were negative included normal adrenal cortex (five cases) (Fig. 1A), placenta (four), pineal gland (one), melanomas (four), follicular thyroid tumors (six), and papillary thyroid tumors (two). Endocrine tumors with few gran-

ules, such as neuroblastomas and small-cell carcinoma of the lung, showed focal and weak immunoreactivity with antibody LK2H10 in a few cases only; these included two of eight cases of neuroblastoma and two of nine cases of small-cell carcinoma of the lung. Nonendocrine cells and tumors that were negative for LK2H10 reactivity included astrocytes (six cases), neurons (four), ganglion cells (six), muscle (five), sarcomas (12), peripheral nerves (11), and benign neurofibromas (six). The cytoplasmic distribution of the immunoreactivity in some tumors including an ovarian carcinoid suggested that the antibody was reacting with cytoplasmic granules (Fig. 1B). Of a total of 169 normal tissues and tumors examined, 69 endocrine tissues, all with secretory granules, showed immunoreactivity with LK2H10.

LK2H10 immunoreactivity was also present in the adrenal medulla and endocrine pancreas of four 13- to 18-week human fetuses and in the adrenal medulla of monkeys and pigs, but absent in the adrenal medulla of sheep, rabbits, guinea pigs, rats, and mice. The pancreatic islets from monkeys and sheep were also negative for this marker while porcine C cells and pancreatic islets showed reactivity similar to that of human endocrine tissues.

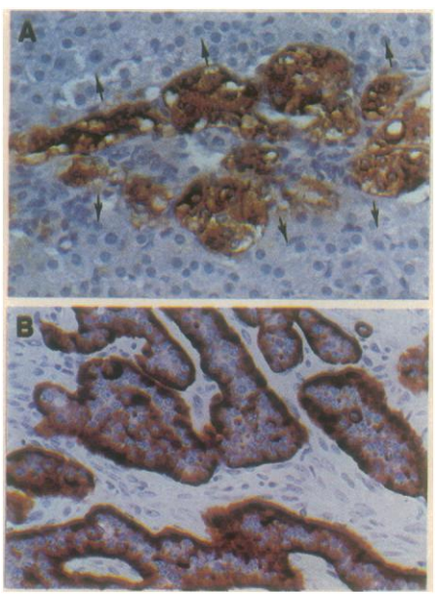

Fig. 1. Monoclonal antibody LK2H10 reactive with endocrine tissues. Normal and neoplastic tissues from Formalin-fixed, paraffin-embedded blocks were stained by the ABC method (*8*). (A) Normal human adrenal tissue shows positive immunoreactivity in the medulla, while the cortical cells (arrows) are negative (×330). (B) An ovarian carcinoid tumor shows positive immunoreactivity, predominantly in the granules located toward the periphery of the tumor cells. The surrounding stromal cells are negative (×330).

To assess the nature of the target molecule detected by monoclonal antibody LK2H10, a pheochromocytoma and a normal adrenal gland from different patients were first extracted with ten volumes of cold phosphate-buffered saline in a Waring blender. The resulting soluble extract was applied to an LK2H10 antibody–Sepharose column and the purified antigen was eluted with 0.5*N* acetic acid. Approximately 0.5 to 1 percent of the added protein was eluted from the affinity column by this procedure. The purified antigen was then electrophoresed in a polyacrylamide slab gel containing sodium dodecyl sulfate (SDS) and the buffer system of Laemmli (*9*). Proteins in the gel were electrophoretically transferred to nitrocellulose paper and then visualized by immunoperoxidase staining with LK2H10. The results showed that LK2H10 reacted with a major band in the pheochromocytoma and normal adrenal of about 68,000 daltons (Fig. 2). If the amount of material in the peroxidase electroblot was increased tenfold for the tumor, additional polypeptides were seen at both higher and lower molecular weights. Because of limiting amounts of material, we were unable to examine the normal adrenal antigen for the additional polypeptides seen for the pheochromocytoma. However, we have recently examined another pheochromocytoma extract and found a complex electroblot pattern similar to that shown in Fig. 2. The relation of the additional polypeptides to the main 68,000-dalton structure is unknown. Preliminary data in our laboratory suggest that they are not a result of proteolysis occurring during the extraction process, since incubation of extracts at 37°C did not generate additional lower molecular weight polypeptides. Thus the higher and lower molecular weight structures reactive with LK2H10 may reflect in vivo processing of this molecule.

To examine whether LK2H10 would react with known hormones, we designed a simple inhibition assay with Formalin-fixed, paraffin-embedded sections. Therefore, a dilution of LK2H10 at which minimum peroxidase staining was observed was allowed to react overnight with various substances before residual immunoreactivity to tissue sections was examined. Although this assay could be blocked by less than 10 ng of the pheochromocytoma antigen purified by affinity chromatography, we found no blocking with 100 μg of endocrine polypeptides of lower molecular weights (bombesin, methionine-enkephalin, glucagon, insulin, secretin, and gastrin) and amines (dopamine, epinephrine, norepinephrine, and serotonin). When the purified pheochromocytoma antigen (100 ng) was used to absorb antibody LK2H10, immunohistochemical staining was blocked in normal adrenal medulla and in a pheochromocytoma.

These results indicate that LK2H10 reacts mainly with a 68,000-dalton polypeptide that is probably associated with endocrine secretory granules, as shown by immunohistochemical staining in granule-containing areas of the cytoplasm in a carcinoid tumor and by poor reactivity with tumors having few granules. The DNE system cells, which are widely dispersed through the body, share such characteristics as dense-core secretory granules and the ability to take

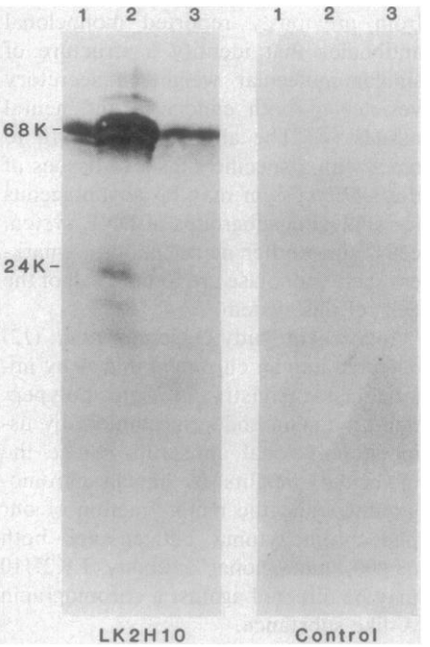

Fig. 2. Molecular weight analysis of the structure detected in human endocrine cells by monoclonal antibody LK2H10. A human pheochromocytoma and a normal adrenal were extracted with ten volumes of cold phosphate-buffered saline (PBS) in a Waring blender at high speed and the resulting soluble extract was passed over a Sepharose 4B column containing LK2H10. The material bound to the column was eluted by 0.5*N* acetic acid, and, after *p*H neutralization, samples were electrophoresed in a Laemmli-type (*9*) SDS-polyacrylamide slab gel (1.5 by 160 mm) at 30 mA. After electrophoresis, the proteins were electrophoretically transferred at right angles (60 V for 4 hours) to nitrocellulose paper by the blot transfer method (*13*). The nitrocellulose paper was then incubated for 1 hour with 4 percent bovine serum albumin in PBS to block nonspecific binding. Subsequent treatments included 1 hour with LK2H10 or a control spent culture medium and 1 hour with peroxidase-conjugated antibody to mouse immunoglobulin G and 10 minutes in diaminobenzidine H_2O_2. Lanes 1 and 2 contain 1 and 10 μg of purified pheochromocytoma antigen, respectively, while lane 3 contains 1 μg of purified adrenal antigen.

up amine precursors and modify them by decarboxylation. Although these cells are not all embryologically derived from the neural crest (*10*), they contain the neuroendocrine marker, neuron-specific enolase (*11*). Not all cells of the DNE system or other cell types with granules are reactive with LK2H10 at the concentrations we used: pancreatic exocrine cells and neurons from the hypothalamus and frontal lobes of the brain and nerve terminals in the posterior pituitary were all negative, as were melanomas. It is not clear whether these cells contain too few granules to exhibit staining or whether they simply do not synthesize the 68,000-dalton substance identified by LK2H10. However, this lack of reactivity with neural tissues distinguishes LK2H10 from previously reported monoclonal antibodies that identify a structure of similar molecular weight in secretory vesicles of both endocrine and neural tissues (*4*). The ability of LK2H10 to react with a specific subset of tissues of the DNE system may be advantageous for analyzing subgroups of DNE system cells, since other neuroendocrine markers such as enolase are found in all of the cells of this system.

In a recent study O'Connor *et al.* (*12*) detected human chromogranin A by immunohistochemistry in eight polypeptide-producing endocrine tumors by using a polyclonal antiserum. Since the molecular weights of human chromogranin A and the major fraction of our pheochromocytoma antigen are both 68,000, monoclonal antibody LK2H10 may be directed against a chromogranin A–like substance.

The availability of large quantities of this monoclonal antibody, which is restricted in specificity to endocrine cells with secretory granules, should permit detailed analyses of normal tissues and tumors of the DNE system from various perspectives. First, it may be possible to analyze the distribution of this antigen in the serum as well as in tumors in patients with neuroendocrine tumors. This antigen can also be used as a marker in Formalin-fixed, paraffin-embedded tissues to diagnose endocrine tumors that have dense-core granules. Second, the development and distribution of neuroendocrine tissues in human fetuses can be analyzed with this marker, especially as they relate to the development of secretory granules in these cells. The embryogenesis of the gastrointestinal endocrine cells, which form part of the DNE system, may be explored more readily with the aid of this marker. Third, the presence of this marker in the adrenal medulla of other animal species, such as monkeys and pigs, will enable investigators to develop and analyze animal models of diseases involving this neuroendocrine tissue.

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Nicarbazin Complex Yields Dinitrocarbanilide as Ultrafine Crystals with Improved Anticoccidial Activity

Abstract. *Nicarbazin, a drug used to control the protozoal disease coccidiosis in poultry, is a complex of the highly insoluble drug 4,4'-dinitrocarbanilide with 2-hydroxy-4,6-dimethylpyrimidine. The structures of this and other 4,4'-dinitrocarbanilide complexes have not been determined, but an analogous 2:1 complex of 4,4'-dinitrodiphenylamine with 1,4-diacetylpiperazine has been prepared in which the only possible bonds are hydrogen bonds between the amide carbonyls and amino hydrogens. Scanning electron microscopy revealed that micron-size crystals of nicarbazin disintegrate in water to form much smaller dinitrocarbanilide crystals. Similar complex dissolution in the gut of poultry may account for the greater effectiveness of dinitrocarbanilide when administered as complexed rather than uncomplexed drug. Particle size problems associated with other highly insoluble drugs and pesticides may be resolved by the use of nicarbazin-like complexes.*

In broiler production, prevention of infection by enteric protozoan parasites of the subclass Coccidia is of critical importance (*1*). Coccidiosis has effects on poultry ranging from reduced feed efficiency to high mortality, depending on the species of invading coccidia, strain pathogenicity, and other factors. The first agent found to give satisfactory broad-spectrum control was nicarbazin (*2, 3*), the 1:1 complex of 4,4'-dinitrocarbanilide (DNC) with 2-hydroxy-4,6-dimethylpyrimidine (HDP) (*4*). This is administered in the feed at a level of 0.0125 percent. Nicarbazin has been used as a coccidiostat for nearly three decades now. However, since the original reports on this drug, the phenomenon of potentiation by complexation which it illustrates has not been investigated to our knowledge.

On a weight basis, nicarbazin is at least ten times as potent as DNC in the control of *Eimeria tenella*, the main cecal pathogen of chicken coccidiosis. In trials with poultry maintained in batteries or pens, approximately the same ratio for potencies of complexed and uncomplexed DNC is observed for five species of *Eimeria* (*E. tenella, E. necatrix, E. brunetti, E. maxima*, and *E. acervulina*). Since HDP has no anticoccidial activity when used alone (*2*), its contribution as a complex component must be associated with improved absorption of DNC. A similar situation prevails with other complexes, such as DNC combined with 1,4-diacetylpiperazine (*5*), 3-methoxy-2(1H)-pyridinone (*6*), and 3-amino-1,2,4-triazine (*7*). The complexes are more effective than DNC and approach nicarbazin in potency, although the noncarbanilide components themselves have no anticoccidial activity.

Complexes of HDP with antibacterial and antifungal ureas, thioureas, and guanidines have been patented (*8*). These are claimed to be more potent than the uncomplexed biocides. For example, the toxicity of the *N,N'*-bis(3,4-dichlorophenyl)thiourea–HDP complex to *Staphylococcus pyogenes* in vitro is reported