Light and electron microscopic characterization of monoclonal antibodies recognizing neuronal cell nuclei and the neurohypophysis
by Harwood John Cranston III

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biological Sciences
Montana State University

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LIGHT AND ELECTRON MICROSCOPIC CHARACTERIZATION OF MONOCLONAL ANTIBODIES RECOGNIZING NEURONAL CELL NUCLEI AND THE NEUROHYPOPHYSIS

by

Harwood John Cranston III

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Date November 25 1986

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ABSTRACT

Since their inception in 1975, monoclonal antibodies (MAbs) have rapidly become the probe of choice for many investigators interested in describing unknown antigens found in complex heterogeneous immunogens. This study characterized five MAbs which bind previously undescribed antigens in the rat nervous and neuroendocrine systems. Immunocytochemical techniques for light and transmission electron microscopic (TEM) examination were employed. MAb F5-26 was reactive with an antigen present on chromatin fibers of nerve cell nuclei in both the central and peripheral nervous systems as well as nuclei of cells found in the adrenal gland and oocytes.

Four MAbs recognizing antigens found in the pituitary gland, 13-11A, 16-5G, 40-5B and 11-1F, were reactive with antigens found in the neural lobe but not in either the intermediate or anterior lobes when examined using indirect immunofluorescent methods. However, an exhaustive TEM study failed to determine which ultrastructural elements the immunoprecipitate was associated with. Further experimentation determined that the solution used to fix the tissue for TEM study had destroyed its antigenicity. Future experimention should include a search for another fixing solution that will enable the tissue to retain its antigenicity.

INTRODUCTION

Monoclonal antibodies (MAbs) are rapidly becoming an indispensible tool for the study of a variety of biological systems. MAbs which are specific for neuronal cell-surface antigens (Levine et al., 1984; Allen and Akeson, 1985; Jacob et al., 1984) have allowed investigators to more closely examine developmental and functional properties which govern cell to cell interactions such as recognition and adhesion which are crucial events during differentiation of the nervous system. Neuronal migration, biochemical differentiation and axonal elongation all may depend on recognition of cell-type specific surface molecules (Kandel and Schwartz, 1982).

The long term goal of this laboratory is to perform developmental and functional studies using MAbs specific for surface molecules of magnocellular neurosecretory neurons of the hypothalamus. As a result of this research, several MAbs have been obtained which recognize antigens other than those found on the cell surface. The present study was undertaken to describe five such MAbs: one recognizes a chromatin associated protein specific to rat neuronal cell nuclei and four are specific for antigens of the rat pituitary gland.

Over the past several years, this laboratory has used the hypothalamo-neurohypophyseal system as a model to examine those properties of a neuron which allow its axon

to locate the appropriate terminating area. This system lends itself to development of MAbs for a number of reasons. The physical boundaries of both the supraoptic nucleus (SON) and paraventricular nucleus (PVN) have been delineated and the cell bodies comprising them can be isolated from whole brain. The axonal pathways of the magnocellular neurons have been extensively described (Silverman and Zimmerman, 1983) and the axon terminals in the posterior pituitary are readily harvested. This system provides an easily isolated, naturally enriched source of membrane from one specific cell type for use as immunogens to produce MAbs.

Because the surface of a cell is covered with thousands of antigens (Sikora and Smedley, 1984) the production of polyclonal antibodies to study individual antigenic determinants in a crude membrane extract of nervous tissue would not be possible. In contrast, MAbs have the abilty to recognize individual antigenic determinants within complex immunogens. The production of cell-type specific MAbs can be equally challenging since it is impossible to predetermine which antigens will be recognized by the monoclonal antibodies obtained. Even using crude membrane preparations produced largely from magnocellular perikarya or axon terminals is no guarantee that the MAbs produced will recognize these structures exclusively. Antigens may be shared by several cell types, non-membrane

antigens likely contaminate the preparation and cell-type specific proteins may possess antigenic epitopes which are shared with many other proteins. In spite of the potential obstacles, the production and characterization of any MAbs specific for a neuronal antigen is potentially important because so few have been developed and because any additions to this library of exquisitely sensitive probes will help us to learn more about neuronal nuclear and pituitary form and function.

Monoclonal Antibody Preparation

MAbs do not differ structurally from naturally occuring antibodies and schematically they may be thought of as Y-shaped molecules with two identical antigen binding sites at the tips of the arms. The N-terminal amino acid sequence located there is specific to the antibody and binds only one antigen. All other antibodies will have different sequences and therefore will be specific for different antigenic epitopes.

An antibody molecule is composed of two identical heavy chains and two identical light chains. Portions of each heavy and light chain participate in the antigen binding site while the heavy chain alone forms the Fc region of the antibody molecule. The Fc region will determine to which of the five classes the antibody belongs as well as its characteristic interaction with other proteins.

Antibodies are produced by B-lymphocytes which have been activated by an antigen. The lymphocyte produced antibody is released directly from the cell into the surrounding tissue fluid. If it were possible to grow this type of lymphocyte in culture, MAbs could be produced very easily. However normal B-lymphocytes do not grow well in culture. Methods first developed in the laboratory of George Kohler and Cesar Milstein (1975) have solved this problem and so have enabled others to produce copious amounts of monoclonal antibody specific for one antigenic epitope. They discovered that a myeloma, a non-secreting malignancy of B-lymphocytes, grows well in culture. The cultured myeloma cells can be fused with an antibody producing B-lymphocyte. The resulting fusion product is called a hybridoma and has retained the characteristics of both parent cells: the rapidly growing non-secreting traits of the myeloma and antibody producing character of the lymphocyte. As a result, the hybridoma can grow forever and produce an unlimited supply of a single monoclonal antibody.

Significance of Nuclear Antibodies

Development of monoclonal and polyclonal antibodies directed against specific enzyme markers (Jones et al., 1980), histone-like proteins (Lutz et al., 1985) and non-histone chromosomal (NHC) proteins (Smith et al., 1978;

Bustin and Neihart, 1979) has advanced our knowledge of nuclear cytoarchitecture and physiology. The production of such exquisitely sensitive probes against other additional nuclear proteins will permit further delineation of the complex nature of the nucleus.

Preliminary light microscopic and immunoblotting experiments revealed that one of the MAbs included in this project recognizes a neuronal nuclear protein (Cranston, et al., 1985) having a molecular weight of approximately 42,000 daltons (Hapner and Paden, 1984). This MAb, F5-26, may bind to one or more of the proteinaceous components of the nucleus. A definitive characterization of this MAb requires a successful electron microscopic localization. Immunoperoxidase methods, which produce an electron dense reaction product, will be employed to localize the antibody binding antigen.

Several easily identifiable structures are apparent in an electron micrograph of a neuronal nucleus. A very clear nuclear envelope is apparent and its double membrane can be easily distinguished by the perinuclear cisterna. The nucleus is filled with chromatin material and one or more nucleoli. Immunoprecipitate associated with any of these structures will appear as black electron dense specks which stand out against the more electron lucid background of nucleoplasm.

The large size of the antigen protein recognized by Mab

F5-26 makes it very unlikely that it is a histone or "high mobility group" (HMG) protein as described by Johns (1982). Larger non-histone chromosomal, enzymatic and structural proteins or proteins associated with the nuclear envelope are more likely candidates. Thus the current state of knowledge regarding these proteins will be briefly reviewed.

Membrane, pore, and laminar proteins are contained in the nuclear envelope which is composed of two typical lipid bilayer membranes separated by the perinuclear cisterna. Pore elements span the perinuclear cisterna joining both membrane layers and it is speculated that they are composed of a single polypeptide with a molecular weight of approximately 68,000 daltons (Hancock and Boulikas, 1982). The lamina, located between the inner membrane and the most peripheral chromatin, is 80 to 300 nm thick in different cell types, and is interrupted at the pores (Hancock and Boulikas, 1982). Enzymatic digestion and ultrastructural cytochemical experiments indicate that it is primarily composed of a protein known as lamin. Richardson and Maddy (1980) using solid state lactoperoxidase labelling, have shown that the pore-lamina component of rat liver nuclear envelope is approximately 93% protein, 6% RNA, and 1% phospholipid.

Enzymes isolated from nuclei have been shown to act upon DNA, RNA, histones, and non-histone chromosomal pro-

teins (Weisbrod, 1982). A number of these enzymes act to modify the molecular composition of chromosomal proteins and are necessary for accurate gene expression.

Most of the nucleoplasm is filled with chromatin, the deoxyribonucleoprotein (DNP) material of chromosomes. The chromatin fiber consists of a chain of repeating subunits called nucleosomes (Noll, 1974). The nucleosome is a particle of 160-170 base pairs of DNA associated with all five histones, two each of H2A, H2B, H3, and H4 found in the core and a single molecule of histone H1 attached to "linker" DNA (Cartwright et al., 1982; Weintraub, 1985). Weintraub (1984) has shown that histone H1 cross-links inactive regions of chromatin in such a way as to hold together ajacent nucleosomes. A complimentary study conducted by Schissel and Brown (1984) demonstrated that in vitro removal of histone H1 from the linker DNA of inactive Xenopus oocyte 5S genes leads to active transcription.

The chromatin fiber exhibits three different levels of organization. The 100Å fiber, described above, gives chromatin the familiar "beads on a string" appearance. It is believed that nucleosomes are further organized by histone and NHC proteins into 300Å coils containing 6-7 nucleosomes arrayed in a helical manner (McGhee et al., 1980). Finally, the 300Å coil is organized into a superhelical macrostructure. It has been suggested that the

torsional strains put on B-DNA by this supercoiling make it hypersensetive to transcription (Weisbrod, 1982; Mayfield et al., 1978) and factors involved in transcription may require a non-B-DNA form to be activated. Alternatively, proteins such as histone H1, which are involved in repression of gene expression, may bind weakly to this superhelical template thus allowing transcriptional events to occur (Weintraub, 1985).

Though many of the mechanisms involved in repression and derepression of gene activity are understood (Weisbrod, 1982; Weintraub, 1985) there remains much that we do not know. NHC proteins play an integral role in these events yet the number of completely described species remains small. It seems quite possible that MAb F5-26 could recognize such a protein and if so characterization of that protein may contribute to the understanding of the control of gene activity.

The function of NHC proteins can be characterized according to their distribution along transcriptionally active sites, their time of action in the transcription process or according to whether their function is enzymatic or structural (Cartwright et al., 1982). Several NHC proteins have been extensively characterized and include protein A24, H1°, IP-25, "high mobility group" (HMG) and "low mobility group" (LMG) proteins.

Protein A24 is a complex of ubiquitin and histone H2A.

Of all H2A found in the nucleosome approximately 10% is complexed in this manner. The precise role of protein A24 is unclear but it has been shown that it is associated with inactive sequences (Goldknopf et al., 1978).

Proteins $H\overset{\circ}{1}$ and IP-25 are similar to histone H1 in structure and appear at times to occupy the linker DNA region (Keppel et al., 1979). Though their function in the nucleosome is unclear, studies have suggested that protein $H\overset{\circ}{1}$ may replace histone H1 in association with linker DNA (Smith and Johns, 1980).

It has been estimated that there are approximately 10,000 to 100,000 molecules of NHC proteins per nucleus of mammalian cells (Johns, 1982). HMG proteins are by far the most abundant and most thouroghly characterized of all NHC proteins. The terms "high mobility group" and "low mobility group" are used to describe the relative movement of these proteins on polyacrylamide gels (Goodwin et al., 1973).

Heizmann (1980, 1982) and Kuenzle (1983, 1984) detected fluctuations in the concentration of protein 35K, a "low mobility group" NHC protein of neurons with a high affinity for single-strand DNA. Those fluctuations coincided with the arrest of differentiation and mitotic division in rat cerebellar granule cells. In 1984, Kuenzle's experiments produced neuroblastoma cells in culture in which the concentration of protein 35K did not fluctuate either when

the cells were left to divide or were induced to differentiate. Homologous proteins present in other cell types demonstrated no change in concentration with respect to artificial manipulation. From these studies it appears that protein 35K can be assigned no specific role but likely serves a structural rather than catalytic function.

A review of the literature reveals that Kuenzle's study may be the only work that has been conducted concerning identification and characterization of NHC proteins which are specific to neurons. This study will likely expand our knowledge of neuron specific proteins and possibly provide the basic groundwork required for further understanding of the molecular mechanisms of neuronal gene control.

Significance of Pituitary Antibodies

The cellular and subcellular localization of MAb binding and subsequent purification of the antigens recognized will undoubtably lead to a greater understanding of the organization, function and control of the posterior pituitary. Four MAbs included in this study, 13-11A, 11-1F, 16-5G and 40-5B, recognize antigens in the posterior lobe.

The magnocellular neurosecretory system has provided a model to describe the principles of neurosecretion for many years (Silvermann and Zimmerman, 1983). These cells, located in the SON and PVN of the hypothalamus, produce

the polypeptides oxytocin and vasopressin, package them in membrane bound neurosecretory granules, and then transport them by axoplasmic flow to terminals in the posterior pituitary for release into the general circulation. Oxytocin is a nonapeptide capable of stimulating uterine contraction and milk letdown. Vasopressin, another nonapeptide, stimulates water absorption in the distal renal tubules and has been shown to stimulate vascular contraction of smooth muscle in arterial vessels (Altura and Altura, 1984; Penit et al., 1983). In addition, vasopressin has been implicated in the improvement of memory (Jolles, 1983) and has been shown to influence neuronal firing rate within various regions of the central nervous system (Tanaka, et al., 1977).

The cytoarchitecture and physiology of the posterior pituitary have been extensively studied by many investigators (for review see Cross, 1983). Pituicytes, a type of astroglia cell, comprise 25 to 30% of the total volume of the rat posterior pituitary (Tweedle, 1983). Their physiological role may include phagocytosis of degenerating axons (Dellman and Rodriquez, 1970; Tweedle, 1983) or control of hormone release from axon terminals into the circulation (Tweedle, 1983). A number of control mechanisms for hormone release have been suggested, and include (1) release of GABA from pituicyte processes surrounding terminals, which inhibits hormone release (Beart et al.,

1974), (ii) pituicyte regulation of the extracellular ionic environment (Dellman et al., 1974) and (iii) prevention of axon terminals from contacting the basement membrane (Tweedle, 1983). In the latter case, it was shown that the extent of nerve ending contact on capillary basement membrane increased during periods of parturition. This suggests that at times of increased hormone demand pituicyte foot processes, separating axons from the basement membrane, draw back allowing an increase in functional axon-basement membrane contact area.

Blood vessels of the posterior pituitary are generally fenestrated, which allows the hormone entry into the circulation. Livingstone (1975) reported that moderate stimulation of hormone release from the posterior pituitary is accompanied by increased blood flow and vasodilation. Chronic dehydration in young rats leads to a significant increase in the proliferation of capillary endothelial cells (Paterson and Leblond, 1977).

Nordmann (1977) has classified the neural elements of the posterior pituitary into, (i) axonal swellings (non-terminal dilatations, containing neither microvesicles or neurotubles), (ii) axonal endings (terminal dilatations, containing microvesicles but no microtubles) and (iii) axons (undilated, containing neurotubles but no or very few microvesicles). Because of the extensive axonal surfaces contained within it, the posterior pituitary would

provide a naturally enriched source of membrane from which to isolate neurosecretory surface antigens for use as immunogens to produce MAbs. Because the system is so well understood it seems ideally suited for the study of cell-type-specific surface antigens.

METHODS AND MATERIALS

Preparation of Monoclonal Antibodies

The mouse monoclonal antibodies characterized in this study were first produced by Paden and Hapner (1983) in this laboratory using a variation of a hybridoma technique developed by Galfre and Milstein (1981). Immunogens consisted of crude membrane extracts fixed with picric acid + formaldehyde (Zamboni's A fixative) and isolated from either adult Holtzmann rat posterior pituitary gland or paraventricular nucleus (PVN). Table 1 summarizes the immunogens and immunization procedures employed.

MAb	Immunogen	Immunization Procedure
13-11A	adult fixed post. pituitary	in vitro activation using immunogen suspended in culture medium.
11-1F	"	"
40-5B	"	"
16-5G	"	ip injection of immunogen followed after 10 wks. by in vitro activation with original membrane prep.
F5-26	adult fixed PVN	ip injection of immunogen followed after 7 wks. by in vitro activation with original membrane prep.

Table 1: Summary of immunization procedures.

After an incubation period of 10-14 days, activated splenocytes were fused with myeloma cells (cell line X-63), resuspended in culture medium and dispensed into culture plate wells. Hybridoma antibody production was detected using an enzyme linked immunosorbent assay (ELISA) test (Parker, 1983) and positive wells were subsequently screened using indirect immunofluorescence staining methods on vibratome cut sections of rat brain tissue. Hybridomas producing antibody that showed good staining specificity on fixed whole pituitary or brain tissue were further processed to produce an ascites fluid. Pristane, an irritating substance, was injected into the peritoneal cavity of BALB/C mice 7 days prior to the injection of $1x10^{6}$ hybridoma cells. Ascites fluid was tapped several times during the 7-14 days of tumor growth. The serum was allowed to clot and was then centrifuged to remove erythrocyte and collagen contamination. Finally, antibiotics were added and the fluid was stored at 4° C.

Following collection of ascites fluid, optimal working dilutions for immunocytochemistry were determined for each tap (ascites fluid dilution experiments for MAb F5-26 were conducted before this study began, therefore my experiments were confined to pituitary specific MAbs only). Adult Holtzmann rats were perfused transcardially for 5 minutes with a saline rinse then 9 minutes with Zamboni's A fixative (4.6% formaldehyde + 15% saturated picric acid

in .1M sodium phosphate buffer pH 7.3). The brain and whole pituitary were removed and post-fixed in the same fixative overnight at 4° C. Tissues were then embedded in Tissue-Tek O.T.C. Compound, cooled to -20° C and sectioned (15 um) in a cryostat at -20° C. Sections were thaw-mounted and air dried on gelatin-chromium potassium sulfate (subbed) treated slides. Sections were stained with an indirect immunofluorescence staining procedure (see appendix).

Ascites fluids collected at different times which demonstrated similar optimal working dilutions were pooled, diluted 1:10 in PBS, sterile filtered and stored at 4° C.

Experimental Approach for Determining the Staining Characteristics of MAb F5-26

To ascertain the location of the antigen recognized by MAb F5-26, both nervous and non-nervous tissues were examined. Brain, heart, kidney, liver, smooth muscle, skeletal muscle, adrenal gland, ovary and testis were examined by light microscopy to determine specificity to different non-nervous tissues. Normal mouse ascites fluid (NS1) was used as a negative control to determine background levels of nonspecific staining. Fixation and perfusion techniques used were as previously described and tissue sections were cut at 50 um on a vibratome. A combination of fluorescence and peroxidase immunocytochemical techniques were employed to examine central nervous system (CNS) versus peripheral nervous system (PNS) specificity. To enhance the specific

staining while reducing background, the avidin-biotin complex (ABC) method of immunoperoxidase staining, developed by Hsu et al., (1981) and obtained from Vector Inc., was employed (see appendix).

The survey of CNS and PNS structures was conducted using the ABC immunocytochemical procedure as described in the appendix. Areas of the CNS examined included hippocampus, reticular formation, thalamus, hypothalamus, caudate/putamen, nucleus accumbens, amygdala, several cranial nerve nuclei and spinal cord. Areas of the PNS examined included superior cervical ganglia, dorsal root ganglia, Auerbach's plexuses and Meissner's plexuses. The small size of these tissues required that they be embedded in egg albumin before being cut into 50 um sections on a vibratome and stained using the ABC method.

Indirect immunofluorescence techniques were used to determine if all neurons possess the antigen recognized by MAb F5-26. Fixed tissues were taken from Holtzmann rats and included cerebral cortex, cerebellum, brainstem, cervical, thoracic and lumbar spinal cord and dorsal root ganglia.

Transmission electron microscopy (TEM) was used to localize the antigen at the ultrastructural level in adult Holtzmann rat cerebral cortex. In order to best preserve tissue ultrastructure, fixation of tissue for this study used a 4% paraformaldehyde + .05% glutaraldehyde + .02%

calcium chloride (P/G) solution (Oldfield et al., 1984).

Because background staining is not a serious problem in TEM studies, pretreatment with normal goat serum is not required. To enhance penetration of the primary antibody without severely damaging the cytoarchitecture, .02% saponin replaces .1% triton X-100 in the primary antibody solution (Oldfield et al., 1984). Tissue sections were stained using the same ABC immunocytochemical procedures described for light microscopy. In addition, after the DAB reaction procedure has been completed, specimens were further processed for TEM examination by osmification, dehydration and embedding of the tissue in Spurr's resin prior to being cut on the ultramicrotome (see appendix).

Experimental Approach to Determine the Staining Characteristics of Pituitary Specific MAbs

Experiments were designed to determine which of the MAbs showed specific, consistent and repeatable electron microscopic staining patterns.

MAbs 13-11A, 11-1F, 16-5G and 40-5B were applied to Zamboni's A fixed adult rat whole pituitary tissue, using both the indirect immunofluorescent and ABC immunocytochemical procedures previously described, and examined in the light microscope. These experiments were used to determine MAb specificity, and to some extent ultrastructural localization of antigen within the pituitary tissue alone. Ascites fluid from myeloma cell line X-63 and

normal mouse ascites (NS1) were used as negative controls to establish background staining levels.

A variety of tissues were examined to determine if the antigens recognized by these MAbs are restricted to pituitary tissue. P/G fixed adult rat liver, kidney, brain, heart, skeletal muscle and smooth muscle were examined. Immunofluorescence methods applied to cryostat tissue sections and ABC technique using vibratome sections were used.

Finally, a study of the ultrastructural localization of the staining seen in the light microscope was undertaken. MAb 40-5B was dropped from this series of experiments because of poor staining characteristics observed in the light microscope. The TEM procedures used were the same as those employed to examine MAb F5-26. In addition, two variations of the original protocol were used to enhance the penetration of primary MAb into tissue sections. Sections were incubated in 50% ethanol with three-ten minute changes followed by six-ten minute rinses in .1M NaPO4 as a pretreatment prior to incubation of tissue in primary antibody. In this experiment .02% saponin was eliminated. The second variation calls for the addition of .02% saponin to all reagents and rinses up to and including the avidin-biotin complex. This step is followed by several vigorous rinses in saponin-free .1M NaPO4 buffer prior to incubating the tissue sections in the reagent containing

Ethanol Pretreated Tissue

MAb	13-11A		16-5B		11-1F	X-63 ascites	
Tissue	Ant.	Post.	Ant.	Post.	Post. only	Ant.	Post.
#caps cut	2	6	0	2	2	3	5
#caps exam.	2	2	0	2	2	3	5
#pos. caps	0	1	0	1	0	0	0

Saponin Treated Tissue

MAb	13-11A		16-5G		11-1F	X-63	
Tissue	Ant.	Post.	Ant.	Post.	Post. only	Ant.	Post.
#caps cut	0	1	0	0	0	3	3
#caps exam.	0	1	0	0	0	3	3
#pos. caps	0	0	0	0	0	0	1

Original Saponin Protocol

MAb	13-11A		16-5G		11-1F	X-63	
Tissue	Ant.	Post.	Ant.	Post.	Post. only	Ant.	Post.
#caps cut	0	6	0	8	8	3	3
#caps exam.	0	6	0	8	8	3	3
#pos. caps	0	4	0	1	1	0	2

Table 2: Summary of TEM tissue preparation and examination. Tissue samples were prepared with three different TEM protocols then were cut and examined. Tissues were prepared by either pretreatment in 50% ethanol, addition of .02% saponin to all immunocytochemical reagents and rinses up and including the ABC reagent or using the original protocol. The number of Beem capsules containing tissue that stained is also listed.

DAB. Table 2 provides a summary of the number of Beem capsules containing tissue samples that were prepared using the three different protocols.

Analysis

Light microscopic examination of tissue sections stained with immunofluorescence and immunoperoxidase methods were examined with a Zeiss Photomicroscope and an Olympus BH-2 light microscope. Transmission electron microscopic examination and photographing of ultrathin tissue section grids was done with a Zeiss EM10/CR electron microscope operated at 60kV with a typical range of magnification of 1200x to 15,000x.

RESULTS

MAb F5-26 Light Microscopic Evaluation

The distribution of MAb F5-26 staining of nervous and non-nervous tissue revealed by light microscopy is summarized in Tables 3 and 4. Neurons in all areas of the central and peripheral nervous systems which were examined exhibited immunoperoxidase staining localized in the nucleus, but not the nucleolus, and to a lesser and more variable extent the cell cytoplasm (fig.1). The appearance of the reaction product differed with different fixation procedures. The reaction product present in the nuclei of rat brain tissue fixed in Zamboni's A fixative appears dark brown and agranular. Brain tissue fixed in paraformaldehyde + glutaraldehyde (P/G) presents a slightly darker and more punctate appearance. Staining of cytoplasm appeared much weaker and little or no difference was seen in tissue fixed with either solution.

As shown in Table 4, the only staining observed in non-nervous tissue was in the adrenal gland and oocyte (fig.2). As with nervous tissue, nuclear staining was evident in both while the nucleolus remains clear. Cytoplasmic immunoreactivity may be present, but is hard to differentiate from background staining.

The adrenal gland shows variation in staining within

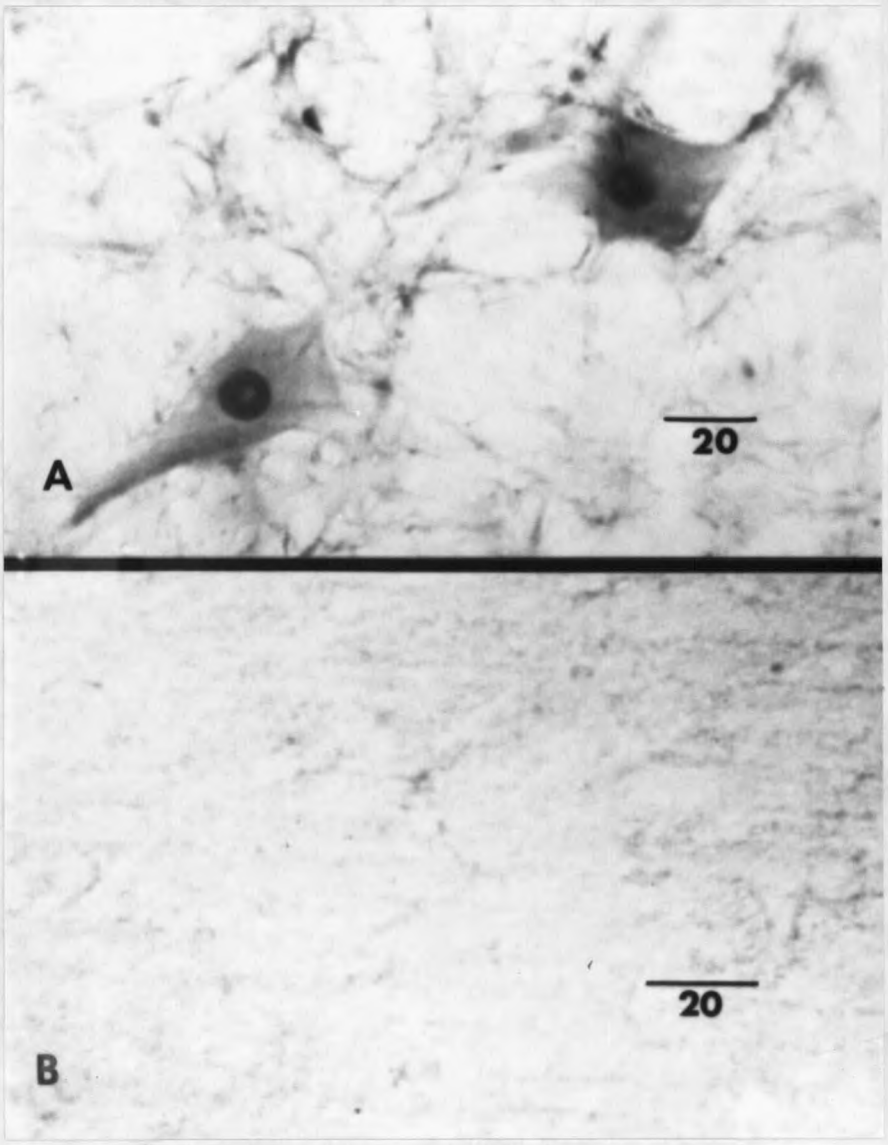

Figure 1: Light micrograph of MAb F5-26 immunoperoxidase staining of thalamic neurons. Micrograph (a) shows the darkly staining nucleus, clear nucleolus and lightly staining cytoplasm. Negative control micrograph (b) is thalamic tissue incubated in NS1 (normal mouse ascites) at the same dilution. Tissue fixed in Zamboni's A; ABC immunoperoxidase method; scale bar in um.

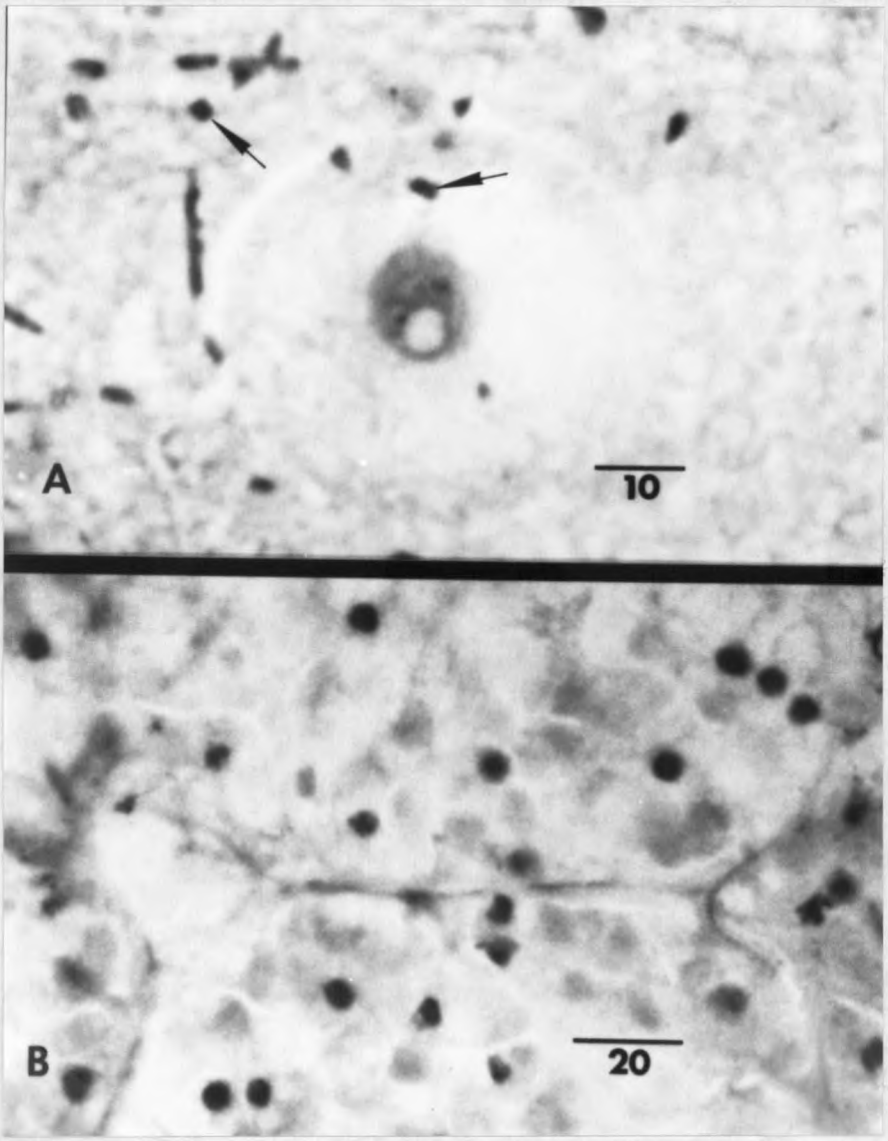

Figure 2: Light micrograph of MAb F5-26 immunoperoxidase staining oocyte (a) and adrenal cortex (b) cell nuclei. Note non-staining nucleolus in oocyte and several staining erythrocytes (arrows) due to endogenous peroxidase activity. Tissue fixed with Zamboni's A solution; ABC immunostaining method; scale bar in um.

Central Nervous System

- cerebral cortex
- thalamus
- caudate/putamen
- globus pallidus
- amygdala
- nucleus accumbens
- central grey
- hippocampus
- reticular formation
- cerebellum
- cranial nuclei 5,7,10,12
- medulla
- spinal cord

Peripheral Nervous System

- superior cervical ganglion
- dorsal root ganglion
- Auerbach's plexus
- Meissner's plexus

Table 3: CNS and PNS areas exhibiting nuclear and cytoplasmic immunostaining with MAb F5-26. Tissue fixed with Zamboni's A solution and stained with ABC immunoperoxidase methods.

Tissue type	Staining	
	Nuclear	Cytoplasmic
heart	-	-
kidney	-	-
liver	-	-
smooth muscle	-	-
skeletal muscle	-	-
testi	-	-
ovary-oocyte	++	-
granulosa cells	-	-
adrenal gland		
zona glomerulosa	+++	+
zona fasciculata	+	+
zona reticularis	-	-
medulla	++	+
pituitary-anterior	-	-
posterior	-	-

Table 4: Distribution of MAb F5-26 immunostaining of non-nervous tissue. Tissue fixed with Zamboni's A solution; ABC immunoperoxidase method.

the cortex as well as between cortical and medullary regions. Within the cortex, the zona glomerulosa demonstrates the greatest number of stained cells followed by zona fasciculata which shows relatively fewer positive cells. The zona reticularus, examined in several tissue sections, showed few or no stained cells. The adrenal medulla also contained immunopositive cells.

Within the ovary, the only cells stained were oocytes in secondary follicles. Staining of nuclear material was quite clear though cytoplasmic staining was difficult to detect above non-specific background.

The experiments designed to determine whether or not all neuronal nuclei are immunoreactive failed for technical reasons. This study involved cutting 15um sections of tissue from both the central and peripheral nervous systems with the cryostat. All tissues examined were obtained from Holtzmann rats and fixed in Zamboni's A solution. Tissues examined showed neither nuclear or cytoplasmic staining regardless of whether fluoroscein or ABC procedures were used. This would seem to indicate that the antigen specified by MAb F5-26, or other associated proteins, are susceptible to denaturation when exposed to temperatures of -20° C followed by thawing.

In summary, all subpopulations of nerve cells examined in the central and peripheral nervous systems demonstrated staining in both nuclei and cytoplasm. There was no

indication that any non-neural elements of the brain or spinal cord exhibit specific staining above background levels seen in negative controls.

Transmission Electron Microscopic (TEM) Staining Characteristics of MAb F5-26

Large neurons from the cerebral cortex had peroxidase reaction product associated with chromatin fibers (fig.3a). The precipitate was not associated with every strand of chromatin, but rather with a few strands distributed throughout the nucleus. Staining of the cytoplasm was not evident in the electron micrographs.

Pituitary MAb Dilution Experiments

These experiments were designed to establish optimal working dilutions for pituitary specfic MAbs. The results indicated that two working dilutions were optimal: 1:500 and 1:250 for tissue fixed in Zamboni's A and examined in the light microscope, and a more concentrated 1:50 dilution for P/G fixed tissue to be examined by TEM. One exception to the latter concentration is MAb 13-11A which was diluted 1:250.

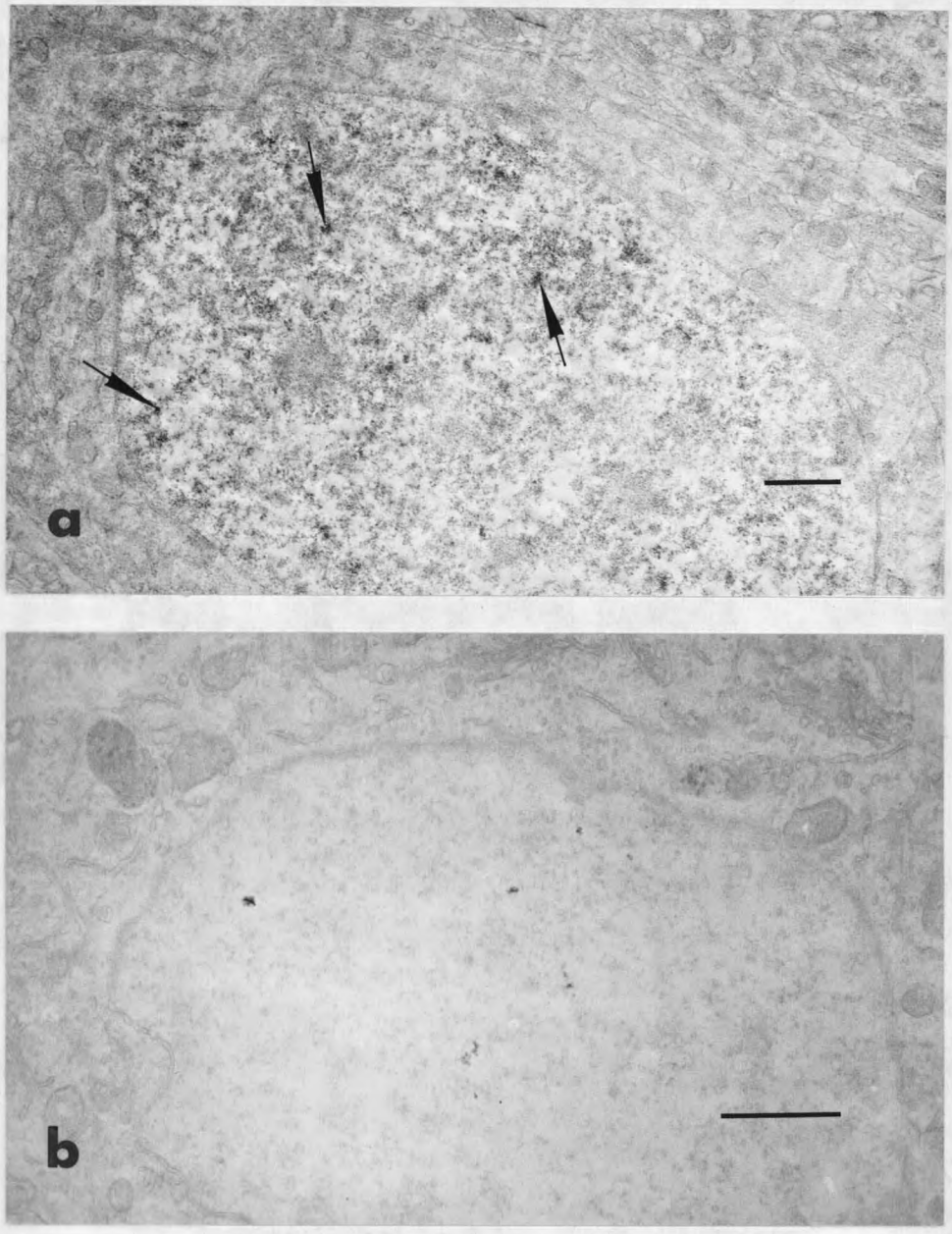

Figure 3: Electron micrograph of MAb F5-26 staining (a) and negative control (incubated in NS1 normal mouse ascites) non-staining (b) cortical neurons. Electron dense particles indicate the assocation of MAb F5-26 with chromatin fibers (arrows). The absence of immunoprecipitate in 3b indicated that staining in 3a was the result of MAb F5-26 association with the antigen. Tissue fixed in P/G; ABC immunoperoxidase method; scale bar equals 1 um.

Light Microscopic Examination of Zamboni's A and P/G Fixed Pituitary Tissue

The first series of experiments involved the staining of 30 um thick sections of Zamboni's A fixed pituitary and brain tissue with hybridoma culture supernatant and ascites fluid. A summary of the staining patterns observed is presented in Table 5. The ascites fluid and culture medium supernatant for each MAb demonstrated similar staining characteristics. MAbs 11-1F and 16-5G as well as ascites fluid from myeloma line X-63 had the greatest specificity to posterior pituitary tissue. MAbs 13-11A and 40-5B showed somewhat weaker staining in the posterior pituitary. Anterior pituitary lobe staining was weak for all MAbs and X-63 ascites fluid. The intermediate lobe was not stained by any of the MAbs or X-63 ascites fluid and normal mouse ascites fluid (NS1) was negative on all tissues.

All MAbs and X-63 supernatant showed some degree of cytoplasmic staining of brain neurons while NS1 did not.

Table 6 summarizes light microscopic staining specificity of MAbs, X-63 ascites fluid, and normal mouse ascites fluid directed at P/G fixed pituitary and brain tissue (figs. 4 and 5).

In general the precipitate was darker and more granular on tissue fixed with P/G. Vacuolated structures were noted in posterior pituitary tissue and was attributed to the

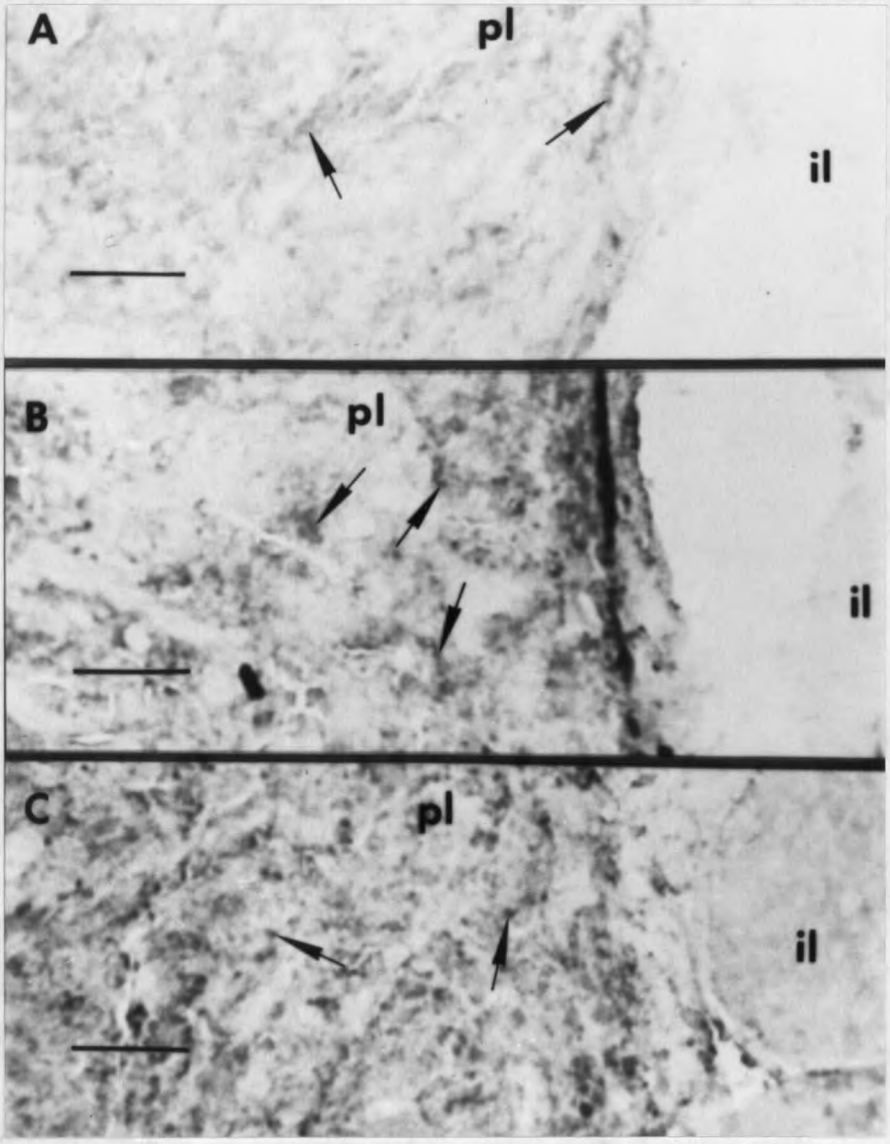

Figure 4: Light micrograph of pituitary tissue. Posterior lobe (pl) and intermediate lobe (il), immunostained with MAb 16-5G (A), MAb 11-1F (C) and X-63 ascites fluid (B). Note that immunoperoxidase staining (arrows) is restricted to the posterior pituitary lobe. Tissue fixed in P/G solution; ABC immunoperoxidase method; tissue sections 30 um; scale bar equals 30 um.

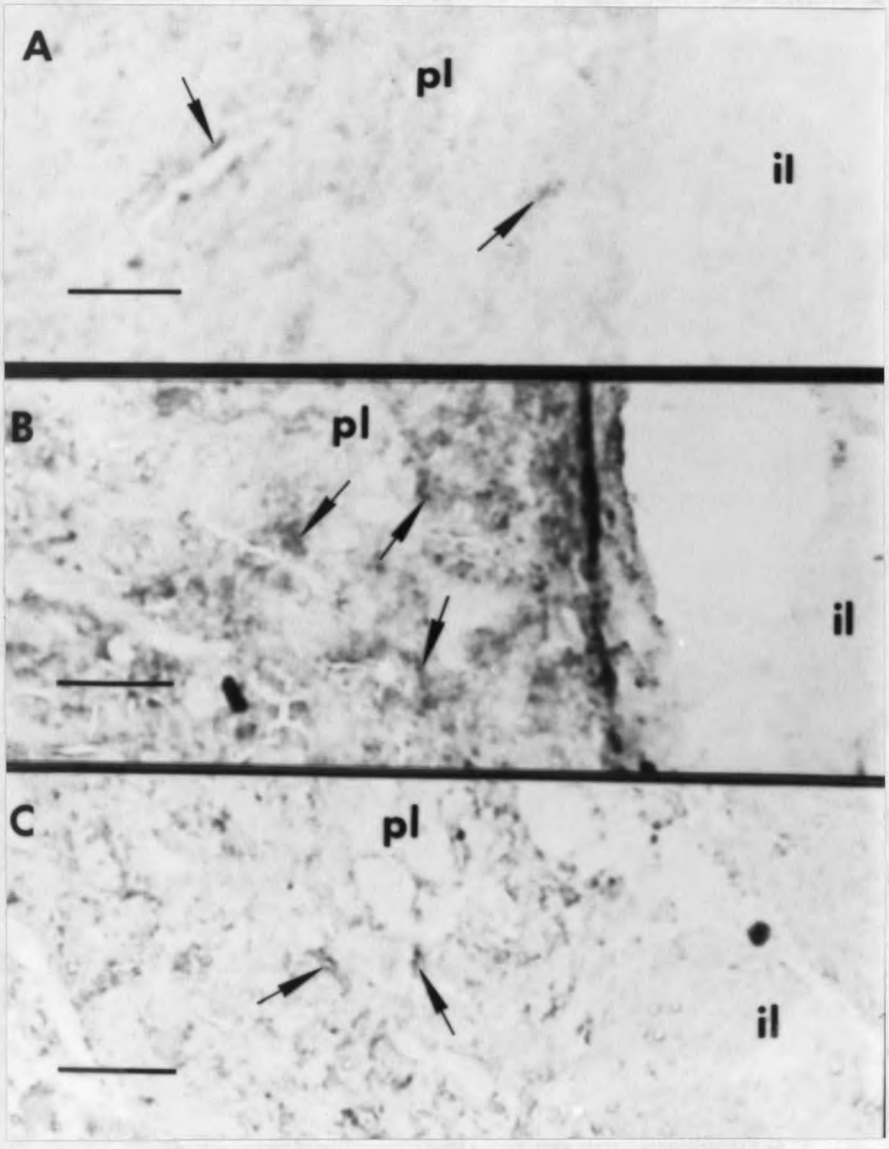

Figure 5: Light micrograph of pituitary tissue. Posterior lobe (pl), and intermediate lobe (il) immunostained with MAb 40-5B (A), MAb 13-11A (C) and X-63 ascites fluid (B). Note that immunoperoxidase staining (arrows) is restricted to the posterior lobe. Tissue fixed in P/G solution; ABC immunoperoxidase method; tissue sections 30 um; scale bar equals 30 um.

MAb	Posterior	Inter.	Ant.
13-11A	filigree background with some punctate groups throughout; some staining around capillaries.	-	Weak cytoplasmic staining; moderate background staining.
16-5G	positive staining around capillaries and breaks in tissue.	-	"
11-1F	weak amorphous staining across entire lobe.	-	some staining around holes in tissue.
40-5B	"	-	weak amorphous staining.
X-63(*) ascites	heavy filigree background.	-	-
super.	-	-	-
NS1(*)	-	-	-

Table 5: MAb specificity to Zamboni's A fixed posterior, intermediate (inter.) and anterior (ant.) pituitary lobes. ABC immunoperoxidase method; supernatant and ascites fluid. (* normal mouse controls).

interaction of TEM immunocytochemical reagents with lipid droplets located within pituicytes. Again, X-63 ascites fluid produced a heavy filigree staining pattern within the posterior pituitary and no staining of intermediate or anterior lobes. NS1 treated tissue did not stain. It was assumed that the similarity in staining seen in tissue fixed with both solutions indicated that the immunogen's antigenicity was maintained. In addition, the intensity of background staining of P/G fixed posterior pituitary tis-

sue was concentration dependent. Intensity of background staining decreased with increasing dilutions of ascites fluid (1:50 to 1:500) in all cases.

Non-nervous tissue fixed with P/G solution was also examined, using each MAb applied to cryostat tissue sections and immunofluorescence staining techniques. Adult Holtzmann rat liver, kidney, heart, skeletal muscle and smooth muscle were examined and no staining was detected for any of the MAbs. Zamboni's A fixed posterior pituitary

MAbs	Posterior	Inter.	Ant.
13-11	vacuolated structures; some pituicyte nuclear staining; variable background.	weak cytopl.	weak nuclear staining and weaker background.
16-5G	vacuolated structures; some other weakly staining structures.	-	"
11-1F	weak amorphous	-	weak amorphous staining.
40-5B	weak amorphous with vacuolated structures.	-	-
X-63(*) ascites only	heavy filigree background.	-	-
NS1(*)	-	-	-

Table 6: MAb specificity to P/G fixed posterior, intermediate (inter.) and anterior (ant.) pituitary lobes. ABC immunoperoxidase method; light microscopic examination; ascites fluid and culture medium supernatant; (* normal mouse controls).

cryostat sections incubated in MAb 11-1F were used as a positive staining control.

Selection of Pituitary MAbs for TEM Examination

Light microscopic examination of P/G fixed posterior pituitary tissue incubated with MAbs 13-11A and 16-5G demonstrated sufficiently intense staining to include them in the TEM study. MAb 11-1F, though showing weak ABC staining of posterior pituitary tissue fixed in this manner, was also included in this study due to its strong and specific staining on Zamboni's A fixed tissue in immunofluorescence experiments. MAb 40-5B, which demonstrated only weak staining, was eliminated from the study.

TEM Examination of Pituitary Structures Associated with Peroxidase Reaction Product

Neither anterior or intermediate lobes of the pituitary showed any signs of reaction product with any of the MAbs utilized. The staining of posterior pituitary tissue was of three types: (i) precipitate associated with the luminal membrane of fenestrated endothelial cells (fig.6), (ii) staining associated with perivascular cell foot processes (fig.7) and (iii) staining associated with the tattered edge of tissue sections. MAbs 13-11A, 16-5G and X-63 ascites showed immunoreactivity on pituitary tissue prepared with the original protocol that included endothelial, perivascular and edge staining (table 7). TEM

MAb	Posterior	Anterior
13-11A	endothelial, perivascular and edge staining	not examined
16-5G	edge staining	no staining
11-1F	endothelial and edge	not examined
X-63(*)	endothelial,perivascular and edge	no staining
NS1(*)	no staining	no staining

Table 7: MAb specificity to P/G fixed pituitary tissue. Tissue prepared with the original TEM protocol; ABC immunostaining method; ascites fluid; (* normal mouse controls).

verified immunoperoxidase reaction product on tissue incubated in X-63 ascites fluid. This result, in conjunction with light microscopic examination that showed strong staining of posterior pituitary by X-63 ascites fluid (table 6), suggested that the TEM staining observed with different MAbs was artifactual. Because staining could possibily be restricted to the tissue surface, the possibility was considered that the observed staining was analogous to an edge artifact. To insure interaction of MAbs with any antigens located below the surface of the tissue, two variations of the original TEM protocol were used to enhance primary antibody penetration.

The first variation called for the addition of .02% saponin to all reagents and rinses. This change lead to additional staining of posterior pituitary incubated in

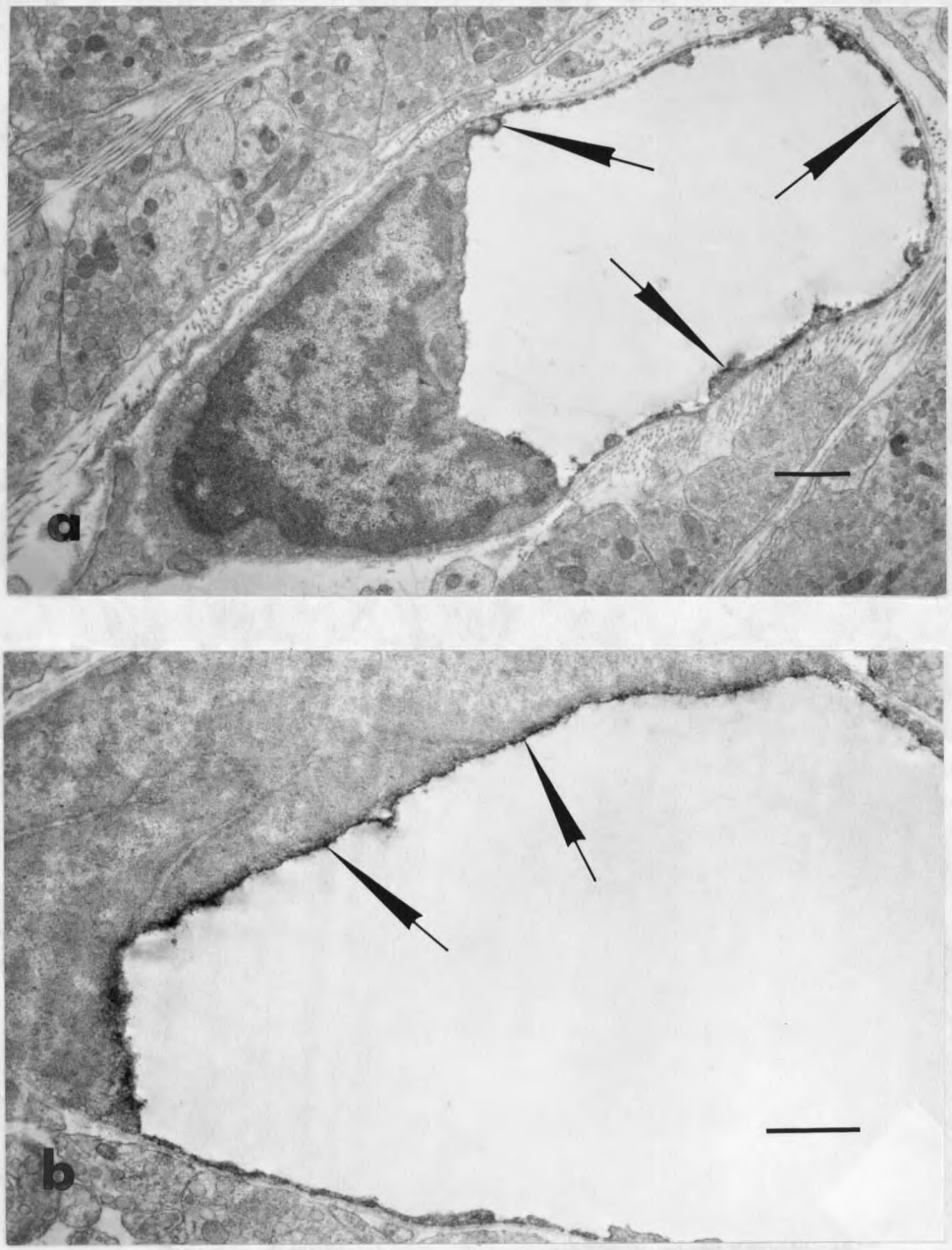

Figure 6: Electron micrograph of pituitary tissue incubated in MAb 13-11A. Micrograph (a) shows precipitate associated with the luminal membrane of fenestrated capillary endothelial cells in the posterior pituitary (arrows). Tissue incubated in X-63 ascites fluid (b) also shows immunoprecipitate associated with the luminal surface (arrows). ABC immunoperoxidase method; tissue fixed with P/G; ascites fluid; scale bar equals 1 um.

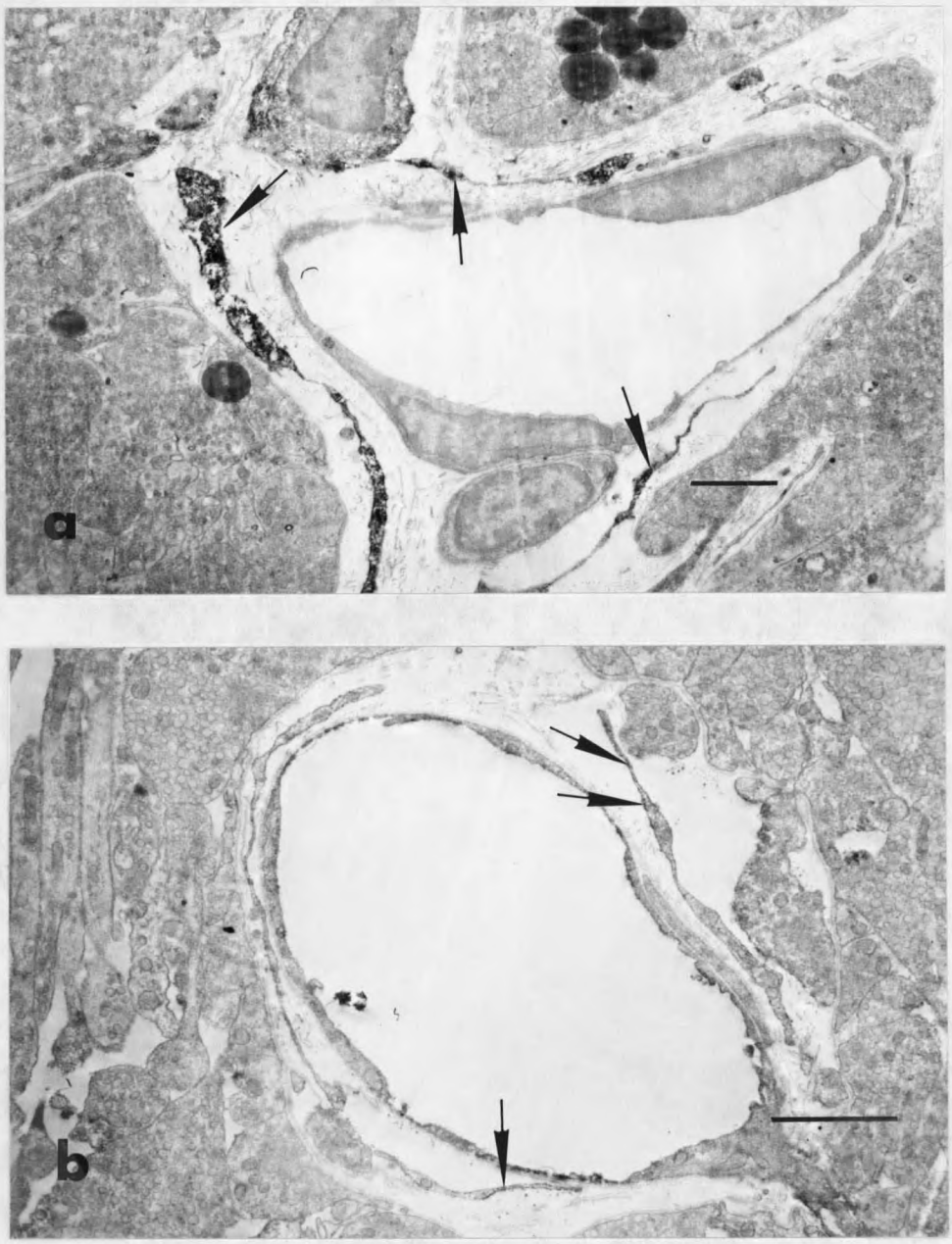

Figure 7: Electron micrograph of pituitary tissue incubated in MAb 16-5G. Micrograph (a) shows reaction product (arrows) associated with perivascular cell foot process in posterior pituitary tissue. Pituitary tissue incubated with X-63 ascites fluid (b) also shows precipitate associated with perivascular cell foot processes (arrows). Tissue fixed with P/G; ABC immunoperoxidase method; ascites fluid; scale bar equals 2 um.

MAb	Posterior	Anterior
13-11A		
saponin	endothelial, perivascular and edge	not examined
ethanol	edge	no staining
16-5G		
saponin	endothelial, perivascular and edge	not examined
ethanol	"	"
11-1F		
saponin	endothelial, edge	"
ethanol	no staining	"
X-63(*)		
saponin	endothelial, perivascular and edge	no staining
ethanol	no staining	"

Table 8: MAb specificity to pituitary tissue prepared with .02% saponin in all reagents and rinses or pretreated with 50% ethanol. P/G fixed; ABC immunoperoxidase method; ascites fluid; TEM examination; (* normal mouse control).

MAb 16-5G and to a general increase in background staining.

The second change in the protocol called for elimination of .02% saponin from primary antibody solutions and pretreatment of tissue sections with ethanol washes (Liewellyn-Smith et al., 1983). Though this procedure dramatically reduced background staining while increasing damage to cellular ultrastructure, it did not reveal any new staining patterns (see table 8).

In summary, examination of posterior, intermediate and anterior lobes of the pituitary with the TEM and using the procedures described above failed to reveal any MAb specific reaction product. Upon reaching this conclusion, an additional experiment was conducted to determine if the P/G fixative destroyed the antigenicity of the molecules recognized by MAbs 13-11A, 16-5G and 11-1F.

MAb	Posterior		Intermediate		Anterior	
	(Z)	(P/G)	(Z)	(P/G)	(Z)	(P/G)
13-11A	++	-	-	-	-	-
16-5G	++	-	-	-	+	-
11-1F	+++	-	-	-	+	-
40-5B	-	N/A	-	N/A	+	N/A
X-63	-	-	-	-	-	-
NS1	-	-	-	-	-	-

Table 9: Distribution of indirect immunofluorescence staining on Zamboni's A fixed (Z) and P/G fixed whole pituitary tissue sections. All sections were incubated in primary antibody prepared with ascites fluid. N/A indicates that the tissue was not examined.

The three MAbs were applied to 50 um cryostat sections of Zamboni's A and P/G fixed pituitary tissue and compared with the light microscope using the indirect immunofluorescence method. The results are summarized in Table 9 and indicate that the P/G solution did indeed severely reduce the antigenicity of the tissue. Tissue fixed with Zamboni's A exhibited the identical distribution of reaction

product as previously described while P/G fixed tissue did not stain.

DISCUSSION

ABC immunoperoxidase staining and light microscopic examination of MAb F5-26 binding in the CNS and PNS showed consistently repeatable staining of neuronal cell nuclei, but not nucleoli, and cell cytoplasm of all areas examined and was restricted to nervous tissue. Non-nervous tissue did not stain positive with the ABC method, with the exception of nuclei of oocytes and adrenal cortical and medullary cells. A review of the literature regarding neuronal nuclear proteins failed to reveal any proteins exibiting such a restricted and unique distribution.

The staining characteristics of MAb F5-26 on cryostat tissue sections were also unique. F5-26 provided the most consistent staining pattern of all the MAbs that were examined in this study. This held true only for vibratome cut tissue stained with ABC immunoperoxidase and fluorescence techniques while cryostat cut tissue did not stain when treated with either technique. Appparently, freezing the tissue at -20° C prior to cutting, followed by thawing and drying of the sections, affects the antigenicity of the tissue. Isolation of the antibody by immunoblotting, which employed unfixed brain tissue homogenate frozen to -20° C, did provide for identification of both the 44.5 and 42kd bands of MAb F5-26 each time it was attempted. This appears to indicate that freezing does not interfere with the antigenic properties of the unfixed protein. It is

possible that fixed tissue responds differently to freezing than does non-fixed tissue. However, it must be kept in mind that the unfixed tissue which exhibited sufficient antigenicity for the SDS gels and western blotting was never dried as were the cryostat sections. Alternative explanations for the loss of immunoreactivity in cryostat sections must include the effect of freezing and thawing on other closely associated proteins. Perhaps these processes change the characteristics of associated proteins in such a way as to mask the antigenic site.

Alternative methods of drying tissue (e.g. in a vacuum or under dry nitrogen gas) may provide clues to the mechanism responsible for this loss of antigenicity. In one experiment, the cut cryostat sections were placed in incubating wells and processed similarly to vibratome sections rather than dried. The result was extremely heavy background, apparently due to extensive tissue damage.

The question of whether the F5-26 antigen is present in all nerve cells remains unanswered. The purpose of the cryostat study was to cut tissue thin enough to determine if all nuclei in the section were stained. Since the conclusion of this study several alternative methods of thin sectioning have been tried by other investigators in this laboratory without success. Thus this problem remains unsolved and must be the focus of future experimentation.

The transmission electron microscopic (TEM) study of

rat cerebral cortex neurons shows that the reaction product of MAb F5-26 is associated with some, but not all, chromatin fibers. However, TEM examination failed to associate the staining with any specific organelle in the cytoplasm despite its localization there in light microscopic studies. This may indicate that the antigen found in the cytoplasm is associated with free ribosomes or simply dispersed freely about the cytosol at a concentration too low to be detected with the TEM methods.

Proteins have been identified that serve structural functions in the higher order organization of chromatin fibers while others act to repress and derepress gene activity. The specific function of the protein specified by MAb F5-26 can not be determined until its exact amino acid sequence is determined and its interaction with surrounding molecules is understood.

SDS gel electrophoresis followed by western immunoblotting was performed by other investigators in our laboratory (Hapner and Paden, 1984) and has revealed two protein bands recognized by MAb F5-26. Analysis of whole brain homogenate produced 44,500 and 42,000 dalton bands while a nuclear fraction from the homogenate revealed only the 42,000 dalton band. Two hypotheses can be formulated to explain this pattern. First, MAb F5-26 may recognize an antigenic determinant present on two different protein molecules having similar molecular weights. Second, the

possibility exists that MAb F5-26 recognizes two forms of the same polypeptide, a precursor form presumably manufactured in the cytoplasm and a mature form which accumulates in the nucleus and associates itself with chromatin fibers. There is evidence that many large precursor proteins destined for membrane bound organelles exist in the cytoplasm for varying lengths of time before insertion into the lumen or membrane of the organelle and subsequent processing into the mature form (Hasilik and Neupert, 1980; Sabatini et al., 1982; Blobel, 1982). "Signal sequences" have been most thoroughly studied in the case of nascent proteins destined for mitochondria or chloroplasts. Many of these proteins are coded for by nuclear genes, synthesized on free polysomes and released into the cell cytoplasm prior to uptake by specific organelles (Chua and Schmidt, 1979; Grossman et al., 1980; Nelson and Schatz, 1979). Upon entry into the organelle the precusor protein is acted upon proteolytically, cleaving the signal or address sequence, and forming the mature protein.

Proteins produced in the cytosol and ultimately destined for the nucleus have been studied. Currently two hypotheses are used to explain accumulation and sequestering of proteins within the nucleus. The first model assumes that proteins diffuse freely across the nuclear membrane and subsequently bind to a nondiffusable substrate within the nucleus (DeRobertis, 1983). The second

model calls for selective transport of proteins across the nuclear envelope (Hall et al., 1984). It is clear that small polypeptide molecules can diffuse across the nuclear membrane. However, the diameter of nuclear pores (45Å) limits the size of molecules capable of passage (maximum 70kd), thus excluding many large nuclear proteins.

It appears possible, then, that the 42,000 and 44,500 dalton bands represent two forms of the immunogen, a mature nuclear form and a larger cytoplasmic precursor form containing a "signal" sequence. In order to test this hypothesis, the amino acid sequence of the two protein bands must be determined to show that the proteins are identical except for the 2500 dalton fragment. Should this be the case, the 2500 dalton difference between the two molecules may contain enough information to function as a "signal" sequence.

Dobberstein and associates (1979) reported that the in vitro synthesis of nascent mouse H2-D histocompatability antigen in the absence of membranes produced a polypeptide 1000-2000 daltons larger than the mature form. This nascent polypeptide was capable of cotranslational insertion into ER membrane and it is inferred that the transient cotranslational insertion signal is contained in this precursor protein. Likewise, Hall (1984) reported that only 13 N-terminal amino acids of yeast nuclear protein

alpha-2 are required to guide B-galactosidase into the nucleus of the Xenopus oocyte. The size of this address sequence is less than the 2500 dalton difference between the two protein bands of the F5-26 specific antigen.

A question which has arisen from this discussion is why would a neuron specific nuclear protein manufactured in the cytoplasm have such specific signal sequences which are cleaved following entry into the nucleus while nuclear proteins in other tissues presumably would contain internal (permanent) signals (DeRobertis, 1983). This difference may be related to the fact that mature neurons do not divide while most other cell types do. Once the protein has entered the neuronal nucleus it can shed the extra sequences because it will not have to enter the nucleus again following mitotic division. Nuclear proteins from other tissues must have the capablility to find and re-enter the nucleus.

Pituitary Monoclonal Antibodies

The primary objective of this study was to determine specificity of the four pituitary MAbs examined. Not unexpectedly, the pattern of staining proved to be dependent on both the fixatives and the immunocytochemical procedures used. The experimental data reveal how minor changes in individual reagents can alter antibody specificity and non-specific precipitate distribution.

Examination of Zamboni's A and P/G fixed cryostat sections, incubated with each MAb and X-63 ascites fluid and stained with immunofluorescent methods, clearly revealed staining of the tissue fixed with Zamboni's A and no staining of tissue fixed with P/G. The X-63 ascites fluid negative control did not stain in Zamboni's A whereas all P/G fixed tissue demonstrated a general non-specific increase in background staining.

These data indicate that P/G fixation alters antigenicity of the tissue sufficiently to render it undetectable under these conditions. Two possible explanations for this phenomenon include destruction or masking of the antigenic site or destruction of fluorescence activity by fixation with P/G. In order to minimize the latter possibility, tissue sections were rinsed extensively in sodium phosphate buffer prior to incubation in the primary antibody. The specific staining of Zamboni's A fixed tissue by MAbs 13-11A, 16-5G and 11-1F under these conditions indicate that the antibodies are capable of recognizing their respective antigenic sites using this experimental approach.

Considered together, the TEM and LM experiments suggest that the staining observed when employing tissue fixed with P/G is artifactual. Pituitary tissue incubated with MAbs 13-11A, 16-5G and 11-1F had immunoprecipitate associated with vascular and perivascular elements as

well as with the tattered edges of tissue sections. The X-63 ascites fluid, used as a negative control, showed a reaction product associated with the same structures.

If, as the fluorescence study suggests, no immunoreactive antigen remains in tissue fixed with P/G, then what is the basis for the LM and TEM staining observed on P/G fixed tissue processed with the ABC method?

One possible explanation for the loss of antigenicity is that the tissue antigenicity is intact and the problem is simply a lack of sufficient penetration of the MAb into the tissue. Attempts were made to enhance penetration of the monoclonal antibodies by including a 50% ethanol pretreatment as well as addition of .02% saponin to some reagents. These changes resulted in decreased background staining of tissue treated with ethanol and an increase in background staining of .02% saponin treated tissue, but no new specific staining was observed.

Although TEM study of the tissue indicated that there is no appreciable increase in antibody penetration with either method, light microscopic staining characteristics were affected by saponin. Brain and pituitary tissue treated with saponin had increased staining intensity of posterior pituitary, corpus callosum, anterior commisure, internal capsule and other structures possessing high axonal density. These data indicate that an interaction of saponin with axonal elements may be a possible factor

responsible for the "specific" staining of posterior pituitary tissue (65-70% of its volume is axonal) when compared to the intermediate and anterior pituitary lobes. An exhaustive TEM study of axonal elements found in the posterior pituitary failed to reveal any specific reaction product other than that associated with tattered membranes. The presence of saponin in reagents may act not only as an aide in antibody penetration but may also mask the more specific staining observed in fluorescence treated cryostat sections fixed with Zamboni's A.

Edge staining is a very common artifact that occurs as a result of damage inflicted upon tissue when it is cut prior to staining. One or more of the reagents and or antibodies appeared to be bound non-specifically to the tattered surface. This kind of staining was noted in negative control sections and similar staining seen in tissue incubated in the pituitary MAbs is regarded as artifact.

Perivascular cell foot process staining, present in tissue stained with MAbs 13-11A, 16-5G and X-63 ascites fluid, was associated exclusively with torn membranes. In fact, there were several instances in which a single process possessed torn sections which stained ajacent to non-staining intact membrane. It seems clear that membrane damage is sufficient to explain the presence of immunoprecipitate on these structutres.

All three MAbs and X-63 ascites fluid stained the luminal surface of endothelial cells, and this may be the result of multiple factors. It is assumed that the luminal surface has access to all reagents and MAbs even if the cells are located deep within the tissue section. Yet not all endothelial cells in a given section stained, nor could this type of staining be observed in every pituitary section examined. Simply stated, the experiments producing this type of staining could not be repeated with any consistency. One explanation for this variablilty could be insufficient rinsing of the tissue sections between steps of the protocol. Lack of proper rinsing could leave reagents in contact with the surface of the cell, resulting in non-specific binding to the structure. Endothelial staining was more common in tissue treated with saponin than in tissue treated with ethanol, suggesting that saponin may be a contributing agent.

Another factor which may contribute to this pattern of staining of endothelial cells is the presence of receptors which recognize the Fc portion of antibodies. Many investigators have documented the presence of such receptors on the luminal surface of the endothelial cell (for review see Ryan and Ryan, 1984) as well as their affinity for various classes of immunoglobulin. Fc receptors are not normally present in great numbers but their activation and proliferation can be induced by cell lysates and various

viral infections (Ryan et al., 1980 and 1981).

One possible scenario would allow for the presence of one or more viral infections in our Holtzmann rat colony. A subsequent proliferation of Fc receptors on all endothelial cells would result in non-specific binding of antibodies, including our MAbs, to the luminal surface and a resulting immunoprecipitate localized there. The endothelial staining observed in the TEM study was restricted to the luminal surface only and was absent from fenestrations.

A number of experiments can be proposed which may help to solve the technical problems implicated by this study. First, a fixative which does not contain glutaraldehyde yet preserves antigenicity and tissue ultrastructure must be found. A first step could be the use of Zamboni's A solution for TEM study even though it is not recommended for electron microscopic use. The ultrastructure may not be well preserved but the possibility remains that ultrastructural localization of the antigen could be accomplished. In addition, it would seem rational to try the original P/G fixative without glutaraldehyde.

A second possibility is the use of periodate-lysine-paraformaldehyde fixative which primarily stabilizes carbohydrate moieties and has been suggested as an alternative to glutaraldehyde fixatives (McLean and Nakane, 1974). The authors claim that it can preserve antigenicity

as well as can paraformaldehyde and ultrastructure as well as can glutaraldehyde. Fixation experiments should screen these fixing solutions on both cryostat and fresh vibratome sections using immunofluorescence methods. TEM fixatives which produce a staining pattern similar to that seen on tissue fixed with Zamboni's A should then be processed using ABC and TEM techniques.

To address the problem of insufficient antibody penetration into tissue sections, two techniques may be employed. First, since tissue incubated with antibody solution that contains saponin has too much non-specific background staining, alternative reagents for enhancing MAb penetration must be found. Triton X-100, used for light microscopic study but generally eliminated from TEM protocols due to the resulting ultrastructural damage, may be worth trying even if the ultrastructure is badly damaged. Another approach to maximize penetration would be the use of post-embedding staining techniques. Several methods are available, of which the Lowicryl method is thought to be the least damaging to antigens (Valentino and Crumrine, 1985). This technique allows for identification of immunoperoxidase reaction product on ultrathin sections and would effectively sidestep the penetration problem. It remains to be seen if the antigenicity of pituitary tissue can be maintained through the rigors of tissue dehydration and embedding employed in this method.

Reduction of the non-specific background staining would be helpful in localizing antigens with the light microscope. Pretreatment of tissue sections with normal goat serum prior to application of the primary antibody may help. In this laboratory pretreatment has effectively reduced non-specific staining on rat brain tissue sections. The present study did not employ this method because it was thought that non-specific staining would not interfer with interpretation of TEM micrographs.

Finally, SDS gel electrophoresis and immunoblotting experiments should be conducted to isolate the proteins specified by these MAbs. Positive results with these experiments would help to establish the specificity of MAbs 13-11A, 16-5G and 11-1F because new MAbs could be raised against the isolated proteins and then pooled for greater LM and TEM sensitivity.

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APPENDIX

Experimental Protocols

Indirect Immunofluorescent Procedure for Light Microscopy

All regents are diluted in .01M phosphate buffered saline (PBS) pH 7.40.

1) rinse slides 2x10' in PBS with agitation.
2) primary antibody diluted 1:10, 1:50, 1:100 and 1:500 in PBS is applied to sections dropwise and incubated at room temperature (RT) for 2 hours.
3) rinse slides 2x10' in PBS with agitation.
4) secondary fluorescein conjugated antibody (Hyclone goat anti-mouse Igs [polyvalent] #E-1081-A), is diluted 1:100 in PBS and applied to sections dropwise, incubate at RT for 60'.
5) rinse sections 2x10' in PBS with agitation and coverslip with 1:1 glycerol/PBS (pH 8.0).
6) examine in fluorescent microscope.

ABC Immunocytochemical Procedure for Light Microscopy

This immunocytochemical protocol for light microscopy uses two buffering systems for rinses and antibody dilution: PBS (pH 7.40) and .1M sodium phosphate buffer (NaPO4), pH 7.40

1) Rinse 50 um vibratome sections 2x20' in PBS
2) Pretreatment:
    a) incubate sections with normal-goat serum at 1:200 diluted in PBS for 60' at RT.
    b) rinse 2x10' in PBS.

3) Primary antibody:

a) incubate sections overnight in primary antibody diluted 1:500 in PBS plus .1% triton X-100 at 4° C.

b) rinse 2x10' in PBS.

4) Secondary antibody:

a) incubate sections in biotin-conjugated goat anti-mouse antibody diluted 1:200 in PBS 60' at RT.

b) rinse 2x10' in PBS.

5) Avidin-Biotin Complex:

a) mix 1:100 avidin reagent 1:100 in .1M NaPO4 followed by an equal amount of biotin reagent B.

b) let stand at RT 30' then incubate sections 90' at RT.

c) rinse 2x10' in .01M NaPO4.

6) Diaminobenzidine (DAB) color reaction:

a) mix .05 gr DAB in 100ml .1M NaPO4; filter in dark.

b) add: .04 gr ammonium chloride
.2 gr B-D-glucose
.0003 gr glucose oxidase

c) incubate sections in dark approx. 60' at RT.

d) rinse 2x10' in .01M NaPO4, (.1M sodium potassium phosphate buffer (NaKPO4) pH 7.25 for

electron microscopic immunocytochemistry).

7) Dehydration:

a) float sections onto subbed slides and let air dry overnight,(or dry on slide dryer at 57° C 2-3 hours).

b) dehydration in graded ethanol:

70% 1x10'

95% 2x10'

100% 2x10'

xylene 2x10'

c) coverslip with Permount

ABC Immunocytochemical Procedure for Electron Microscopy

This immunocytochemical protocol for electron microscopy is the same as the light microscopic protocol previously described with the following changes: .1M NaPO4 buffer (pH 7.40) instead of .01M PBS was used throughout (except where noted below). The following procedure begins after step number 6 of the ABC procedure for light microscopy.

1) Examine tissue sections in the light microscope and dissect areas of interest. Rinse 2x10' in .1M NaKPO4 (pH 7.25) buffer.

2) Incubate sections in 2% osmium tetroxide/potassium ferricyanide prepared in .1M NaKPO4 buffer for 55' at RT. Rinse 2x10' in .1M NaKPO4.

3) Dehydration:

50% ethanol 1x10'

70% " 1x10'

95% " 2x10"

100% " 2x10'

100% propylene oxide (PO) 2x10'

1:2 Spurr's resin/PO 1x30'; stir

2:1 Spurr's resin/PO 1x60'; stir

100% Spurr's resin 1x60'; stir

100% Spurr's resin overnight at 4° C

4) Polymerization:

Place specimens in flat tip Beem capsules filled with 100% Spurr's resin. Heat at 70° C 8 hours.

5) OPTIONAL: Post-ultrathin sectioning counterstain with uranyl acetate (UA) and lead citrate (LC).

a) filter UA (saturated in 100% methanol) into depressed slide.

b) incubate tissue grid 5' at RT.

c) rinse in graded methanol 10 seconds each: 100%,90%,70%,50%,30%,15%,filtered sterile H20.

d) blot excess H20 and let air dry.

e) put 3 drops LC on waxed petri dish containing a few pellets of NaOH.

f) incubate grid speciman side down 3' at RT.

g) rinse 10 seconds each with .02N NaOH (CO2 free) solution.

h) rinse 10 seconds with sterile filtered H20.

i) blot and let air dry.

6) Examine specimen in TEM.