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

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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.

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

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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 rea-
sons. The physical boundaries of both the supraoptic nuc-
leus (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 deter-
minants 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 peri-
karya 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 occurring 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 for-ever and produce an unlimited supply of a single mono-clonal 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 transcrip-
tion may require a non-B-DNA form to be activated. Alter-
natively, 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 spe-
cies 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 ac-
cording to their distribution along transcriptionally
active sites, their time of action in the transcription
process or according to whether their function is enzyma-
tic 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 H1° 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 H1° 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 differen-

tiate. 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 bind-

ing and subsequent purification of the antigens recog-

nized 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) preven-
tion of axon terminals from contacting the basement mem-
brane (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 base-
ment membrane, draw back allowing an increase in func-
tional axon-basement membrane contact area.

Blood vessels of the posterior pituitary are generally
fenestrated, which allows the hormone entry into the cir-
culation. Livingstone (1975) reported that moderate stimu-
lation 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 sur-
faces 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 1×10^6 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 (sub-bed) 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, devel-

oped 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, cer-

vical, 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% sapo-
nin 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 mi-
croscopic 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 immunocyto-
chemical procedures previously described, and examined in
the light microscope. These experiments were used to de-
termine MAb specificity, and to some extent ultrastructur-
al 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 pitui-
tary 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. Sec-
tions 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 Tissue	13-11A Ant.	Post.	16-5B Ant.	Post.	11-1F Post. only	X-63 ascites 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 Tissue	13-11A Ant.	Post.	16-5G Ant.	Post.	11-1F Post. only	X-63 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 Tissue	13-11A Ant.	Post.	16-5G Ant.	Post.	11-1F Post. only	X-63 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.

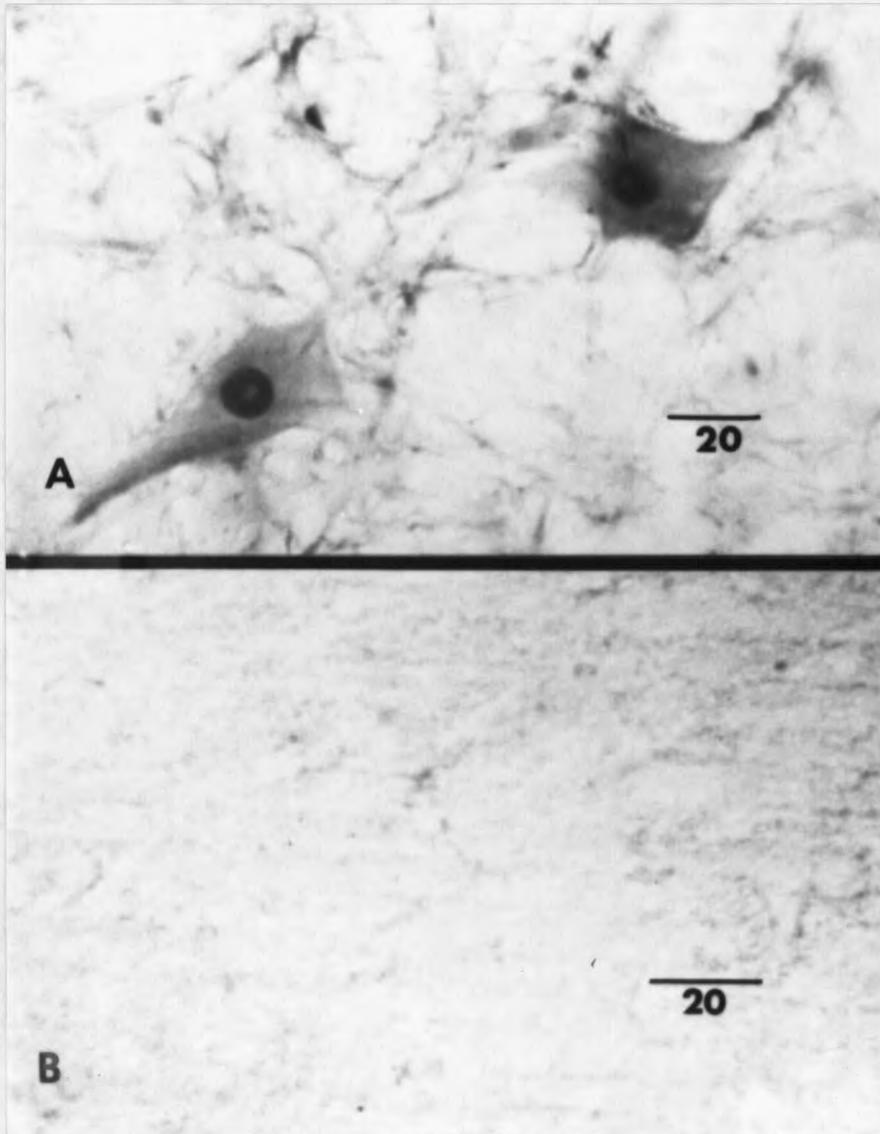The adrenal gland shows variation in staining within

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;
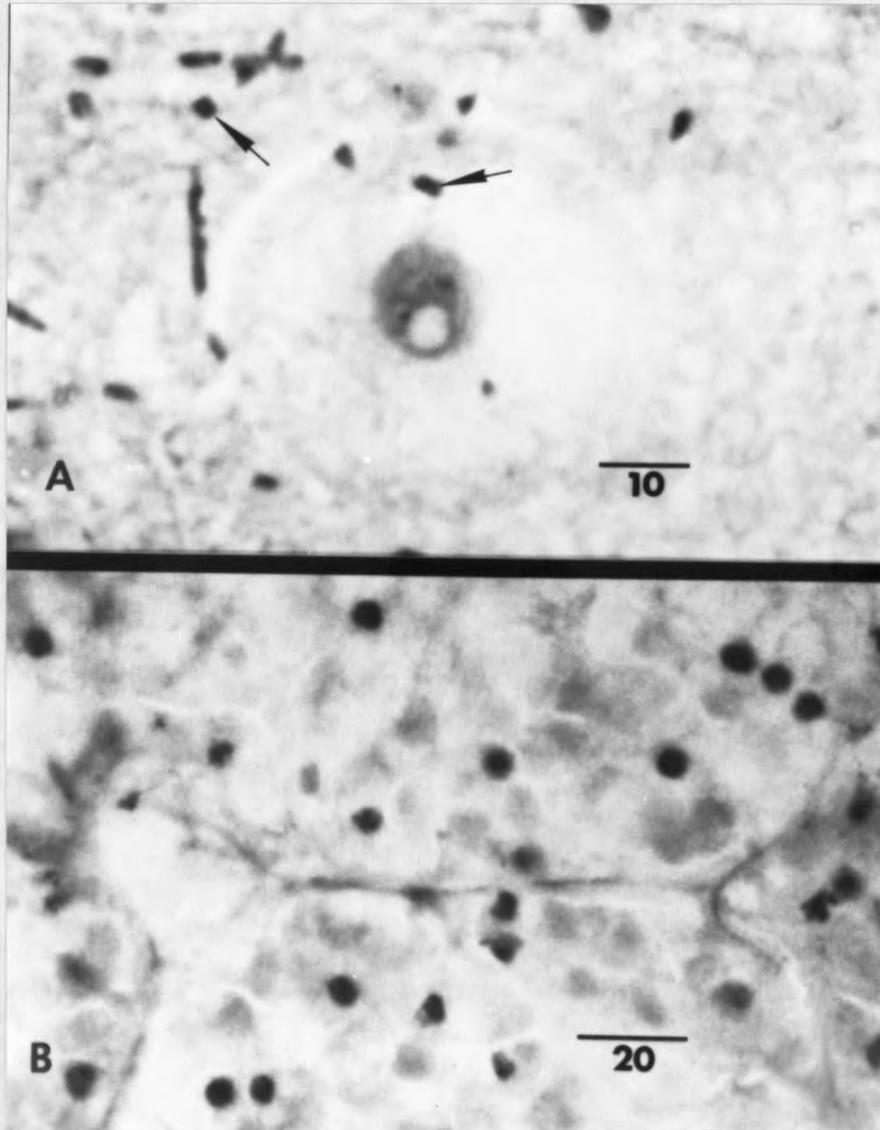ABC immunoperoxidase method; scale bar in um.

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.

| | Staining | |
Tissue type	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.
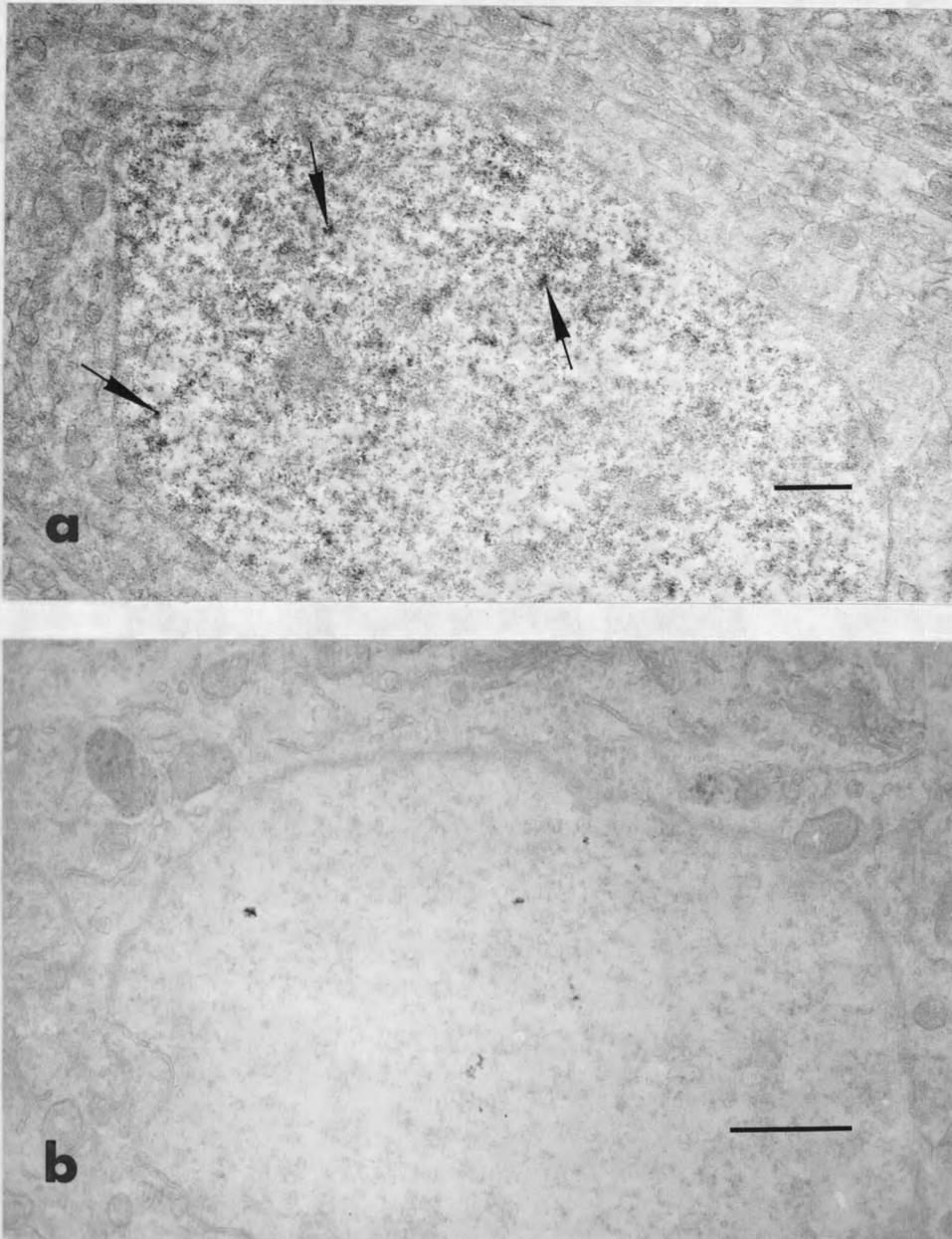
Figure 3: Electron micrograph of MAb F5-26 staining (a) and negative control (incubated in NS1 normal mouse ascites) non-staining (b) cortical neurons. Electron dense particles indicate the assocation of MAb F5-26 with chromatin fibers (arrows). The absence of immunoprecipitate in 3b indicated that staining 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