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MONOCLONAL ANTIBODY TO AN ENDOGENOUS NEUROPEPTIDE
WITH PUTATIVE MORPHINE-MODULATING ACTIVITY

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TECHNICAL REVIEW AND APPROVAL

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The experiments reported herein were conducted according to the principles set forth in the current edition of the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

This technical report has been reviewed by the NMRI scientific and public affairs staff and is approved for publication. It is releasable to the National Technical Information Service where it will be available to the general public, including foreign nations.

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INTRODUCTION

The cardioexcitatory peptide, phe-met-arg-phe-NH₂ (FMRF-NH₂), was originally isolated from ganglia of the mollusc, *marcrocallista nimbosa* (Price and Greenberg, 1977). In molluscs, this peptide was found to have a variety of biological properties including regulatory effects on the heart (Greenberg and Price, 1983), muscle (Painter et al., 1982) as well as modulatory effects on neurons (Colombaioni et al., 1985) and opioid peptides (Greenberg, et al., 1983).

FMRF-NH₂-like peptides have also been detected in CNS of various mammalian species using antisera raised against FMRF-NH₂ (Boer et al., 1980; Weber et al., 1981). Furthermore, in mice and rats, FMRF-NH₂ was found to exert many biological actions including the activity to modulate opiate effects (Kavaliers and Hirst, 1985; Kavaliers et al., 1985; Kavaliers and Hirst, 1986; Tang et al., 1984), excite brain neurons (Gayton, 1982), elevate blood pressure (Mues et al., 1982), induce depolarizing and hypopolarizing responses in cultured mouse spinal neurons, (Guzman et al., 1987) and induce excessive grooming behavior (Raffa et al., 1986). However, the mammalian FMRF-NH₂ immunoreactivity is now known to consist of multiple molecular forms which are not identical to FMRF-NH₂ (Chronwall et al., 1984; Dockray, 1985). Previously, we have isolated and chemically characterized a FMRF-NH₂-like peptide of bovine brain (Yang, et al., 1985). This peptide, phe-leu-phe-gln-pro-gln-arg-phe-NH₂ (F-8-F-NH₂), can attenuate the morphine induced prolongation of tail flick latencies in

the rat when injected intraventricularly (Yang, et al., 1985) and elevate arterial pressure when injected intravenously (Roth et al., 1987). In bovine CNS, F-8-F-NH₂ was found to be highly localized in dorsal spinal cord by radioimmunoassay (Majane and Yang, 1987). In order to further explore the functional role of F-8-F-NH₂, monoclonal antibodies to F-8-F-NH₂ were developed and characterized by radioimmunoassay techniques. The results suggest that these monoclonal antibodies are capable of differentiating F-8-F-NH₂ or F-8-F-NH₂-like peptides from neuropeptide Y (NPY) which is structurally similar to F-8-F-NH₂ or FMRF-NH₂ in its C-terminal sequence. In fact, it is difficult to differentiate FMRF-NH₂-like peptides from NPY immunohistochemically with some polyclonal antisera (Sasek and Elde, 1985).

MATERIALS AND METHODS

Materials:

F-8-F-NH₂, pro-gln-arg-phe-NH₂ (P-4-F-NH₂) and ala-gly-glu-gly-leu-ser-ser-pro-phe-trp-ser-leu-ala-ala-pro-gln-arg-phe-NH₂ (A-18-F-NH₂) were custom synthesized by Peninsula Laboratory, Inc. (Belmont, CA), and purified by HPLC. FMRF-NH₂, met⁵-enk-arg⁶-phe⁷, arg-phe-NH₂ and γ_1 -melanocyte stimulating hormone (γ_1 -MSH) were purchased from Peninsula Laboratory, Inc. Neuropeptide Y (NPY) and peptide YY (PYY) were purchased from Bachem (Torrance, CA).

Immunization of mice:

F-8-F-NH₂, 6 mg, and keyhole limpet hemocyanin, 5 mg, were dissolved in 1 ml H₂O. To this solution, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, 6 mg in 0.1 ml H₂O, was added. After 16 hrs standing at room temperature, the reaction mixture was dialyzed against H₂O overnight and lyophilized. The conjugate was dissolved in saline and then emulsified in complete Freund's adjuvant (Cal. Biochem). Balb/c mice were injected subcutaneously with the F-8-F-NH₂-hemocyanin conjugate, 100 µg in 0.1 ml emulsion, once a week for four weeks. Three days prior to sacrifice, the mouse received the last immunization with the same dose of the conjugate.

Cell fusion and cloning:

Spleen cells (5×10^7) from immunized mice were fused with SP2/0 mouse myeloma cells (5×10^7) at log phase by polyethylene glycol 1500 (Boehringer Mannheim Biochemicals) as previously described (Galfrè and Milstein, 1981). Fused cells were resuspended in 60 ml of RPMI 1640 (Quality Biological, Inc.) culture medium containing hypoxanthine 13.6 mg/l, aminopterin 0.19 mg/l and thymidine 3.875 mg/l (HAT selective medium) and then distributed into 96-well microtiter plates (50 µl cell suspension and 200 µl HAT selective medium/wells). After incubation at 37°C in a CO₂ incubator for 2 days, 200 µl of HAT selective medium in each well was replaced with 250 µl of RPMI 1640 culture medium containing hypoxanthine, 13.6 mg/l, and thymidine, 3.875 mg/l, (HT hybridoma growth medium). Ten days later, tissue culture media from wells with hybridoma

colonies were screened for antibody to F-8-F-NH₂ by enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA). Cells from positive wells were cloned by limiting dilution (Galfrè and Milstein, 1981). Selected cloned hybridomas were preserved in a medium containing 70% RPMI culture medium, 20 % fetal calf serum and 10% DMSO and subsequently used for production of antibodies by tissue culture or by generation of ascitic fluid in mice.

ELISA method:

F-8-F-NH₂, 100 µg, was conjugated to 10 mg of bovine serum albumin (BSA) by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide. The F-8-F-NH₂-BSA conjugate was dialyzed against H₂O overnight and a total of 2 ml was obtained. The F-8-F-NH₂-BSA conjugate was diluted 1:10,000, and then dispensed into each well of microtiter plates (50 µl/well). After incubating at 4° C for 1 hour, wells were emptied, treated with 100 µl 0.5% BSA for 1 hour at 4°C and washed with 3 x100 µl phosphate buffered saline containing 0.04% Triton (PBS-Triton). Spent culture medium that is to be tested was dispensed into duplicate wells (50 µl/well); plates were incubated at 4°C for 1 hr and washed 3 times with 100 µl of PBS-Triton. Goat-anti mouse IgG conjugated to alkaline phosphatase (Sigma) was diluted with PBS-Triton (1:1,000), 50µl of this solution was added to each well and plates were incubated for another hour at 4°C. After repeated (7 times) washings with 100 µl PBS-Triton, 50 µl of p-nitrophenyl phosphate (0.5 mg/ml in 0.05 M Na₂CO₃ and 0.001 M MgCl₂, pH 9.8) was added and

plates were incubated at 37°C for 1.5 hr. The reaction was terminated by the addition of 50 μ l 2 N NaOH into each well and results were read by an ELISA reader (Dynatech).

Radioimmunoassay:

Peptide, tyr-leu-phe-gln-pro-gln-arg-phe-NH₂ (Y-8-F-NH₂) was custom synthesized by Peninsula Laboratory and purified by reverse phase HPLC. Y-8-F-NH₂ was radiolabelled with Na[¹²⁵I] by chloramine T method (McConahey and Dixon, 1980) and purified by BioGel P-2 column chromatography developed with 1 N CH₃COOH containing 0.1% BSA.

For RIA, F-8-F-NH₂ standards or samples were incubated with [¹²⁵I]-Y-8-F-NH₂ (12,000-15,000 CPM) and antibody in 0.5 ml 0.2 M Tris buffer pH 7.4 containing 0.1% BSA, 0.06% dextran (RIA grade, Schwartz/Mann, Spring Valley, NY) in polystyrene tubes overnight at 4°C. Free [¹²⁵I]-Y-8-F-NH₂ was separated from [¹²⁵I]-Y-8-F-NH₂ bound to the antibody by adding 200 μ l of RIA buffer containing 1.5% charcoal. The samples were centrifuged and 0.55 ml supernatants removed and counted in a Beckman biogamma counter.

Isotyping:

The isotyping of the monoclonal antibodies was performed by the sandwich ELISA method with the isotyping kit from Calbiochem (Cat No 386445) following the procedures described by the manufacturer.

Ascitic fluids:

Those hybridomas tested positive in the ELISA were further screened with RIA using [125 I]-Y-8-F-NH₂. The cells of the positive clones were injected (10^6 cells/injection) into the peritoneal cavities of female BALB/c mice primed with 0.5 ml pristane 5 days earlier, and 10-15 days later ascitic fluid was collected.

HPLC fractionation of spinal cord extract:

Bovine cervical spinal cord obtained fresh from a local slaughter house was transported on ice to the laboratory and the dorsal grey region was dissected out. Sprague Dawley rats (200 gms) were decapitated and spinal cords removed. Tissues were homogenized in 10 volume of 1 N CH₃COOH containing 0.02 N HCl by an Ultra-Turax tissumizer (Tekmar, Cincinnati, OH). The homogenates were heated for 10 min in a 100°C water bath and centrifuged at 15,000 x g for 20 min. The pellet was reextracted with 5 volume solution of 1 N acetic acid containing 0.02 HCl. The supernatants from the first and second extractions were combined and passed through a reverse phase C-18 cartridge (Waters Associate, Milford, MA). The C-18 cartridge was washed with 20 ml H₂O and then eluted with 6 ml of 60% CH₃CN containing 0.1% trifluoroacetic acid (TFA). The eluate was lyophilized and redissolved in H₂O for HPLC analysis using an Altex ODS 5 column (4.6 x 250 mm). The reverse phase column was eluted at a flow rate of 1 ml/min with a linear gradient of CH₃CN (20-50%/60 min) formed by CH₃CN containing 0.1% TFA and 0.1% TFA in H₂O. Fractions were collected every min and aliquots

from 1 ml fractions were dried in a vacuum concentrator (Savant, Speed Vac) and radioimmunoassayed with monoclonal antibody containing ascitic fluid. The ascitic fluid was used at a final dilution of 1:275,000.

RESULTS

Production of antibodies:

Three clones of hybridomas secreting monoclonal antibodies against F-8-F-NH₂ were identified after screening with ELISA and RIA and designated X10M1, X13M1 and X30S1. The isotypes of these antibodies were determined to be IgG₁ for X10M1 and X13M1 and IgG_{2b} for X30S1. The ascitic fluids were produced by injecting the hybridomas into the peritoneal chamber of pristane-primed mice. Titers of these three antibodies (ascitic fluids) determined by RIA using [¹²⁵I]-Y-8-F-NH₂ are shown in Fig. 1. Similar titers were observed for all three ascitic fluids. The antibodies (ascitic fluid) at final dilutions of 1:200,000-312,000 were used in RIA.

Antibody specificity:

The specificity of the antibody was examined by inhibition of [¹²⁵I]-Y-8-F-NH₂ binding to monoclonal antibodies by various peptides. The characteristics of the monoclonal antibody, X13M1, is shown in Fig. 2. All of the peptides having arg-phe-NH₂ at their C-termini cross-reacted with the antibody and their rank order of immunoreactive potencies are F-8-F-NH₂ > A-18-F-NH₂ > P-4-F-NH₂ > FMRF-NH₂ > γ_1 -MSH > arg-phe-NH₂. The antibody showed no cross-reaction with neuropeptide Y (NPY), peptide YY (PYY) and met⁵-enkephalin-arg⁶-phe⁷. The relative immunoreactivities of these peptides expressed in (B/Bo)₅₀ (concentrations of peptides required to displace 50% of the [¹²⁵I]-Y-8-F-NH₂ bound to the antibody) is

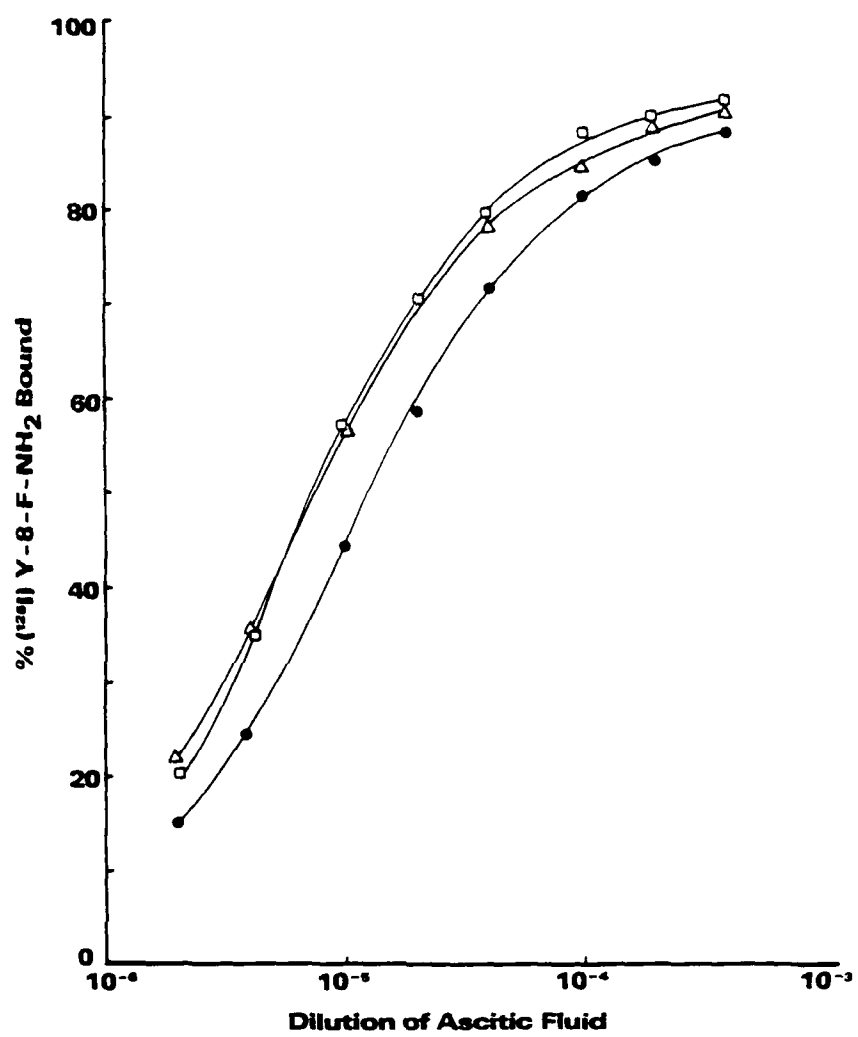


FIGURE 1

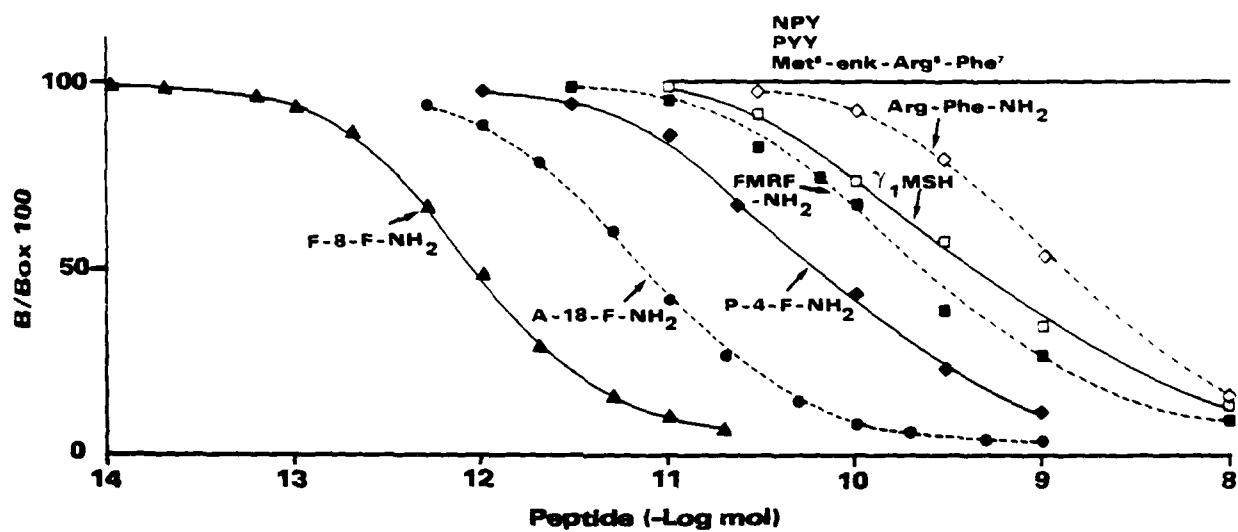


FIGURE 2

summarized in Table 1. Very similar specificities were observed with antibodies X10M1 and X30S1 (data not shown). The limit of RIA sensitivity is 50-100 fmol/sample (Fig. 2) under the RIA conditions used.

RIA of tissue extracts fractionated by HPLC:

To characterize further the monoclonal antibody, extracts from bovine and rat spinal cords were fractionated by reverse phase HPLC and radioimmunoassayed with the monoclonal antibody X13M1. In the bovine spinal cord extract, one major immunoreactive peak eluting in the position of synthetic F-8-F-NH₂ was obtained, although there were some minor immunoreactive peaks (Fig. 3 top). One of the minor immunoreactivities was eluted in the position of synthetic A-18-F-NH₂ and is most likely due to the cross-reactivity of this peptide with the antibody. In the rat spinal cord extract, one major peak and other minor immunoreactivities were obtained. The major peak was eluted with a retention time higher than that of synthetic F-8-F-NH₂ (Fig. 3, bottom).

TABLE 1

Cross-reactivity of F-8-F-NH₂ antibody with structurally related and unrelated peptides

Peptides	Number of Amino acid	Amino acid sequence	(B/B ₀) 50 pmol/tube
F-8-F-NH ₂	8	Phe-Leu-Phe-Gln-Pro-Gln-Arg-Phe-NH ₂	0.9
A-18-F-NH ₂	18	...Ser-Leu-Ala-Ala-Pro-Gln-Arg-Phe-NH ₂	7
PQRF-NH ₂	4	Pro-Gln-Arg-Phe-NH ₂	60
FMRF-NH ₂	4	Phe-Met-Arg-Phe-NH ₂	200
γ ₁ -MSH	11	...Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-NH ₂	500
Arg-Phe-NH ₂	2	Arg-Phe-NH ₂	1000
Met ⁵ -Enk-Arg ⁶ -Phe ⁷	7	Tyr-Gly-Gly-Phe-Met-Arg-Phe-OH	> 10 ⁴ *
Neuropeptide Y	36	...Asn-Leu-Ile-Thr-Arg-Gln-Arg-Tyr-NH ₂	> 10 ⁴ *
Peptide YY	36	...Asn-Leu-Val-Thr-Arg-Gln-Arg-Tyr-NH ₂	> 10 ⁴ *

(B/B₀)₅₀: Amount of peptide required to displace 50% of [¹²⁵I]-Y-8-F-NH₂ bound to the antibody.

*: Peptides which showed no reactivities with the antibody at 10,000 pmol/tube.

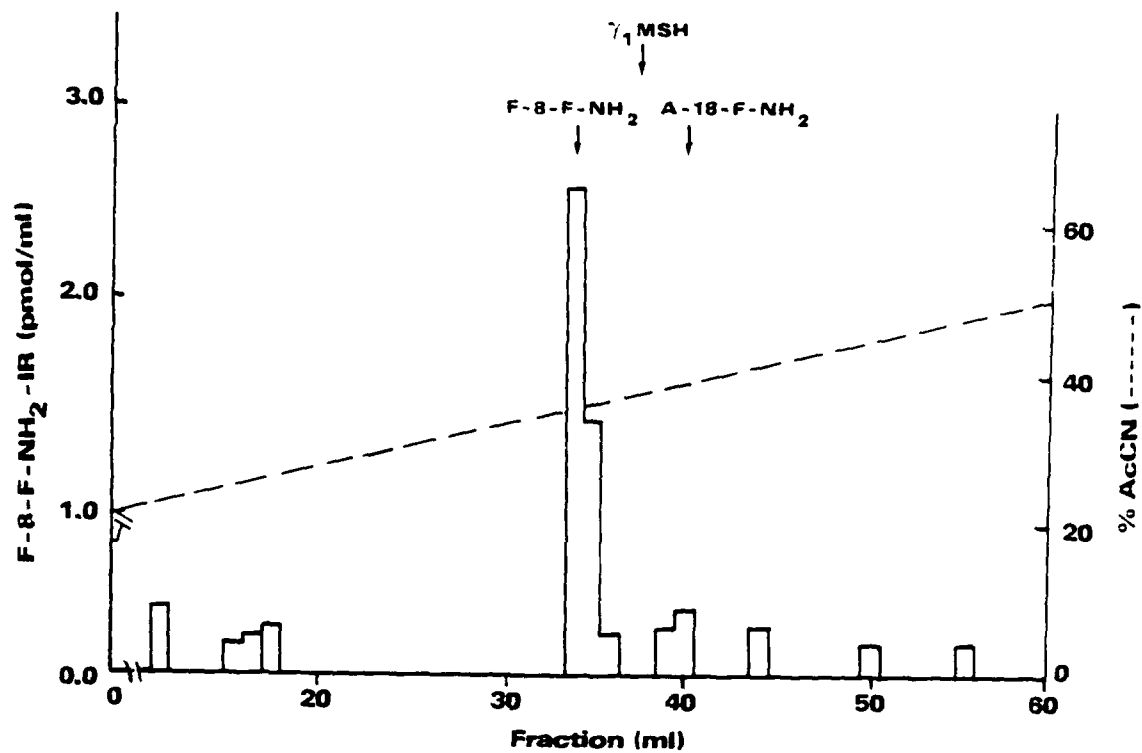


FIGURE 3 (top)

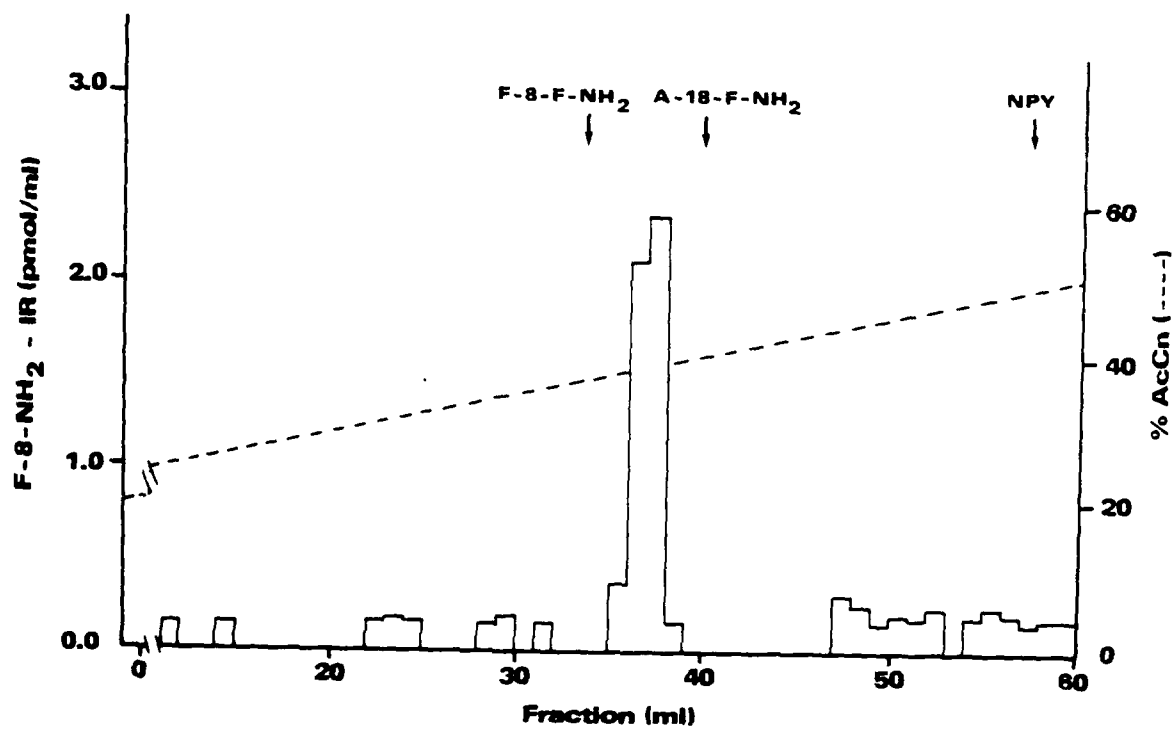


FIGURE 3 (bottom)

DISCUSSION

Three clones of hybridomas secreting antibodies to F-8-F-NH₂ were produced using F-8-F-NH₂ conjugated to hemocyanin as immunogen. From these hybridomas, high titer ascitic fluids were obtained (Fig. 1). The antibodies (ascitic fluid) showed varying degrees of cross-reactivities with peptides which share the same C-terminal dipeptide, arg-phe-NH₂ with F-8-F-NH₂. In contrast, the antibodies were found to be inactive to structurally non-related peptides including NPY. The results suggest that the antibodies are directed to the C-terminal dipeptide arg-phe-NH₂ but the conformation of the peptide appears to also contribute to the immunoreactive potency of the peptide.

The limit of the RIA sensitivity using the monoclonal antibody X13M1 is 50-100 fmol/sample which is 10-20 times lower than the sensitivity obtainable in the RIA with polyclonal antiserum raised in rabbits (Majane and Yang, 1987). In spite of this low RIA sensitivity, ascitic fluids were found to have very high titers (Fig. 1).

Polyclonal antiserum to FMRF-NH₂ or F-8-F-NH₂ has been found to cross-react with NPY weakly (Sasek and Elde, 1985; Majane and Yang, 1987). Because of this and the abundance of NPY in mammalian CNS, it may be difficult, sometimes, to differentiate FMRF-NH₂-like peptides including F-8-F-NH₂ from NPY by immunohistochemical studies with polyclonal antisera (Sasek and Elde, 1985). The monoclonal antibody to F-8-F-NH₂ is unreactive to NPY (fig. 2) and this specificity is further confirmed by the analysis

of spinal cord F-8-F-NH₂ immunoreactivity after the HPLC fractionation. As shown in Fig. 3, the monoclonal antibody seems to detect one major immunoreactivity in the extract from spinal cord of bovine or rat although some very minor immunoreactivities are revealed. The F-8-F-NH₂ immunoreactivity in the rat spinal cord appears to be more hydrophobic than synthetic F-8-F-NH₂ suggesting an interspecies molecular heterogeneity.

The characteristics of the monoclonal antibody to F-8-F-NH₂ produced in this study strongly suggest that this antibody will be very useful in studying the distribution of F-8-F-NH₂ exclusive of NPY in the mammalian CNS. Several lines of evidence suggest that F-8-F-NH₂ may have a modulatory role in opiate mediated analgesia (Tang et al., 1984; Yang et al., 1984 and 1985; Majane and Yang 1987), however, the precise physiological role of F-8-F-NH₂ or FMRF-NH₂-like peptides in mammalian CNS still remain unclear. Immunohistochemical studies on the distribution of F-8-F-NH₂ without interference from NPY, which is highly abundant in the mammalian CNS, may lay a frame work for the further exploration of the role of F-8-F-NH₂ or FMRF-NH₂-like peptide in neuronal function.

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FIG. 1 Titer of the ascitic fluids.

$[^{125}\text{I}]\text{-Y-8-F-NH}_2$ (20,000 CPM) was incubated with various dilutions of ascitic fluids X10M1 (●—●), X13M1 (□—□) or X30S1 (△—△) in 0.5 ml 0.2 M Tris buffer pH 7.4 containing 0.1% BSA, 0.06% dextran in polystyrene tubes overnight at 4°C. Free $[^{125}\text{I}]\text{-Y-8-F-NH}_2$ was separated from $[^{125}\text{I}]\text{-Y-8-F-NH}_2$ bound to antibody by adding 200 μl of RIA buffer containing 1.5% charcoal followed with centrifugation. The result is expressed as % $[^{125}\text{I}]\text{-Y-8-F-NH}_2$ bound to the antibody.

FIG. 2 RIA specificity.

Competitive displacement of $[^{125}\text{I}]\text{-Y-8-F-NH}_2$ bound to monoclonal antibody X13M1 by F-8-F-NH₂ (▲—▲), A-18-F-NH₂ (●—●), P-4-F-NH₂ (◆—◆), FMRF-NH₂ (■—■), γ_1 -MSH (□—□), arg-phe-NH₂ (◇—◇), NPY, PYY and met⁵-enk-arg⁶-phe⁷. The results are expressed as percentage of sample reacted in the absence of competition. B/B₀ is the ratio of the specific binding in the presence of non-labelled peptide to the maximal specific binding.

FIG. 3 High pressure liquid chromatography of F-8-F-NH₂ immunoreactivity in cervical dorsal grey region of bovine spinal cord (top) and rat spinal cord (bottom).

Extracts from 1.5 g bovine cervical dorsal grey or 0.6 g rat spinal cord were concentrated using a C-18 cartridge as described in the text and

applied to an Altex ultrasphere ODS-5 column (4.6x250 mm) eluted with a linear gradient of 20-50% CH₃CN in 0.1% trifluoroacetic acid over 60 min at a flow rate of 1 ml per min. Aliquots from 1 ml fractions were radioimmunoassayed with F-8-F-NH₂ monoclonal antibody X13M1.

ABBREVIATIONS:

CNS,	central nervous system
FMRF-NH ₂ ,	phe-met-arg-phe-NH ₂
F-8-F-NH ₂ ,	phe-leu-phe-gln-pro-gln-arg-phe-NH ₂
NPY,	neuropeptide Y
A-18-F-NH ₂ ,	ala-gly-glu-gly-leu-ser-ser-pro-phe-trp-ser-leu-ala-ala-pro-gln-arg-phe-NH ₂
P-4-F-NH ₂ ,	pro-gln-arg-phe-NH ₂
HPLC,	high pressure liquid chromatography
γ ₁ -MSH,	γ ₁ -melanocyte stimulating hormone
PYY,	neuropeptide YY
HAT,	hypoxanthine, aminopterin, thymidine
HT,	hypoxanthine, thymidine
ELISA,	enzyme-linked immunosorbent assay
RIA,	radioimmunoassay
DMSO,	dimethylsulfoxide
BSA,	bovine serum albumin
PBS,	phosphate buffered saline
Ig,	immunoglobulin
TFA,	trifluoroacetic acid