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Immunology

Monoclonal antibody to the message sequence Tyr-Gly-Gly-Phe of opioid peptides exhibits the specificity requirements of mammalian opioid receptors

(β-endorphin/immunohistochemistry)

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ABSTRACT **Six myeloma cell hybrids producing antibodies to human β-endorphin were isolated from a single mouse spleen. The monoclonal antibodies displayed different binding patterns with the antigen. We report the characterization of one antibody which recognizes the tetrapeptide Tyr-Gly-Gly-Phe representing the message sequence found at the NH_2 terminus of all naturally occurring mammalian opioid peptides. Competition experiments in radioimmunoassay and immunohistochemistry show that the antibody fails to bind the β-endorphin precursor β-lipotropin, does not discriminate among opioid peptides that share the same message sequence but have different COOH-terminal extensions, and does not react with pharmacologically inactive derivatives of β-endorphin. The antibody recognition of the message sequence of natural opoid peptides is sensitive to those molecular changes that affect their receptor binding competence.**

The multihormone precursor pro-opiomelanocortin is a model of a complex post-translational processing that leads to a hierarchy of products with duplicated, overlapping, or divergent biological activities (1–5). The resolution of such polypeptide families requires a combination of biochemical approaches in which antisera are an indispensable tool. However, conventional antisera have inherent limitations which decrease the potential resolving power of the antibody response. The quest for high-titer high-sensitivity antisera is fulfilled by a combination of high-avidity antibodies directed against a few immunodominant determinants at the expense of other epitopes corresponding to potentially more informative areas of the antigen. In addition, the heterogeneity of the antibodies contributes to the variability and range of antigen crossreactivity.

Monoclonal antibodies (6) to a member of a family of genetically related antigens provide a library of reagents individually endowed with uniform, reproducible, combining-site specificity which can discriminate among structurally related polypeptides (7). Moreover, each antibody is essentially a structure probe that can give insights into the fine architecture of the antigen and the conformational differences between polypeptides with homologous or overlapping sequences.

We have applied the monoclonal antibody approach to the analysis of the β-lipotropin (β-LPH) family and related peptides.

EXPERIMENTAL PROCEDURES

Immunogens and Antigens. Human β-endorphin (β_h-EP) (synthetic), [Met]enkephalin, and [Leu]enkephalin were obtained from Beckman (Geneva, Switzerland). All other biopeptides were from Peninsula Laboratories (San Carlos, CA), with the exceptions of human β-LPH and human thyroglobulin which were kindly donated by C. H. Li (University of California, San Francisco) and by H. Gärtner (University of Munich, Munich, Federal Republic of Germany), respectively. The method of Skowski and Fisher (8) for the covalent coupling of β_h-EP to poly-L-lysine (M_r 40,000; Sigma, Munich, Federal Republic of Germany) or to other carrier molecules by using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide·HCl has been described (9).

Radioimmunoassay. Human β-endorphin was iodinated (^{125}I; Amersham Buchler, Braunschweig, Federal Republic of Germany) according to the published protocol (9). In the initial screening of the hybridoma supernatants the unbound tracer was separated by adsorption to charcoal particles (9). However, this method was soon abandoned in favor of the double-antibody precipitation method because it was found that uncharacterized components of the culture supernatants, probably released by the peritoneal feeder cells, occasionally gave rise to false-positive tests. Routinely, 100 μl of hybridoma supernatants was incubated in 500 μl of buffer (10) containing 10^4 cpm of ^{125}I-labeled β_h-EP (specific activity, 300 Ci/mmol; 1 Ci = 3.7×10^{10} Bq). After 16–20 hr at 4°C, 5 μl of normal mouse serum and 6 μl of a goat anti-mouse immunoglobulin antiserum (Paesel, Frankfurt, Federal Republic of Germany) were added and the incubation was continued for about 15 hr. Immunoprecipitates were sedimented at 12,000 × *g* for 4 min and the amount of pelleted tracer was measured with a counting efficiency of 56%. Displacement curves for crossreactivity studies were obtained by using an amount of purified monoclonal antibody (about 5 μg) that bound approximately 20% of the added tracer in the absence of unlabeled peptide.

Immunization of Mice. Prior to the fusion experiments, conditions were sought that would increase the antibody response of the mouse to β_h-EP. Most important was the finding that (BALB/cJBom × C57BL/6JBom)F_1 females consistently gave a 5- to 10-fold higher serum titer than did the BALB/c parent strain. It was also found that changing the macromolecular carrier of β-EP in consecutive immunizations led to higher antibody titers, probably through selection of lymphocyte clones endowed with high-affinity receptors (11–13). The anti-β-EP myeloma hybrids we report here all were derived from the splenocytes of a single F_1 hybrid female. The animal had been

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Abbreviations: β-LPH, β-lipotropin; β_h-EP, human β-endorphin.

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immunized three times (once every 3 weeks) by multiple subcutaneous injections of 100 μg of β_h-EP coupled to poly-L-lysine (0.15 ml emulsified with an equal volume of complete Freund adjuvant). After 8 months from the first immunization, the mouse received a 0.3-ml intraperitoneal injection of 200 μg of β-EP coupled to human thyroglobulin in incomplete Freund adjuvant. Following the suggestions of Stähli *et al.* (14), 3 days later the same immunogen without adjuvants was injected intravenously (200 μg in 0.2 ml of 0.15 M NaCl) and 1 day later it was injected intraperitoneally (100 μg in 0.2 ml of 0.15 M NaCl). Twenty-four hours after the last injection, the animal was sacrificed and splenocytes were prepared for the fusion experiment. The serum was tested in the charcoal radioimmunoassay (9) and found to exhibit 40% binding of ^{125}I-labeled β-EP at 1:600 dilution.

Cell Culture, Fusion, and Cloning. The hybridization procedure used in this work was based on a described technique (15). The cell line BALB/c X63-Ag8.653, a myeloma mutant that fails to produce immunoglobulin chains (16), was used as the transformed fusion partner.

Individual clones were isolated from positive cultures by limiting dilution in microculture plates (Costar 3596) with mouse peritoneal cells used as feeders.

Analysis and Purification of Monoclonal Antibodies. For biosynthetic labeling of proteins, washed cells were incubated for 18 hr in leucine-free, lysine-free minimal Eagle medium (GIBCO) containing 10% dialyzed fetal calf serum and L-[^{3}H]-leucine (New England Nuclear, 110 Ci/mmol) and L-[^{3}H]lysine (New England Nuclear, 64.5 Ci/mmol), each at 30 μCi/ml. Immunoglobulins were purified by affinity chromatography on staphylococcal protein A-Sepharose CL (Pharmacia) as described (17). The ^{3}H-labeled antibodies had specific activities $\geq 2 \times 10^3$ Ci/mmol, in agreement with the results reported by others (7).

The homogeneity of the secreted antibodies isolated by binding to protein A-agarose beads was analyzed by electrophoresis in $NaDodSO_4$/polyacrylamide gels (18).

Serological characterization of immunoglobulins in culture supernatants was performed by a sandwich assay (19). Rabbit anti-mouse immunoglobulin light and heavy chains (Bionetics Laboratories, Kensington, MD) were used in 100 μl volumes to coat the surface of flexible vinyl microtiter plates (Cook 1-220-24B). Mouse immunoglobulin bound to the insolubilized rabbit antibody was detected with a peroxidase-labeled rabbit anti-mouse immunoglobulin serum (DAKO, Copenhagen, Denmark). *o*-Phenylenediamine (20) was used as substrate, and enzyme activity was detected by measuring absorbance at 450 nm in an automatic multichannel photometer.

Immunochemistry. For the immunohistochemical studies of the rat pituitary, the unlabeled antibody method of Sternberger (21) was used. Preparations were obtained from non-colchicine-treated animals and embedded in paraffin as described (22). After treatment with purified immunoglobulin 3-E7 in the absence of detergents for 20–24 hr at 4°C, the sections were washed with phosphate-buffered saline and incubated for 30 min with goat anti-mouse Ig (Paesel). Further treatments were as described (22).

For the immunofluorescence studies, we used frozen sections of formaldehyde-fixed rat brains obtained from colchicine-treated animals as described (23). Purified antibody 3-E7 was dissolved at a concentration of 5 μg/ml in phosphate-buffered saline containing 2.5% (wt/vol) bovine serum albumin and 0.1% Triton X-100. As second antibody, a fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG (Miles) was used.

RESULTS

Detection of β-EP-Specific Hybridomas. All fusion experiments previous to or simultaneous with the one we now report failed to produce β-EP-specific hybridomas. From an estimated population of >2,000 myeloma hybrids obtained with various immunization regimens, routes, and carriers, we accumulated numerous hybridomas with specificity directed against the carrier molecules or even against hybrid determinants but never against β-EP. These results could be explained by the weak immunogenicity of the antigen. Although a single successful experiment can hardly justify generalizations, it should be noted that the finding in this experiment of anti-β-EP antibodies with different binding specificities argues against a straightforward statistical explanation for the failure of the previous hybridizations. Rather, we think that the successful fusion was due to the use of a well-immunized animal that, after a long resting period, had undetectable amounts of circulating antibodies. In these circumstances a higher effective concentration of the injected antigen may lead to a larger size of splenocyte clones at the time chosen for the fusion.

Cells surviving the metabolic selection grew in all culture wells and the supernatants were tested for reactivity against β_h-EP as well as human thyroglobulin, the carrier used only in the last immunization. Thirteen cultures showed binding activity with thyroglobulin (data not shown) and six, with β_h-EP as early as day 13 or 16 after fusion. With the exception of one β-EP hybrid, all others maintained antibody activity during successive dilutions and transfers of the original cultures. However, aliquots of all positive hybridomas were frozen at the earliest detection of antibody activity. The β-EP binding activities of the five stable hybridomas tested in a single displacement radioimmunoassay are shown in Fig. 1. The antibodies manifested different binding patterns for β-EP. Because the myeloma cell parent does not produce immunoglobulin chains that could form hybrid molecules with the anti-β-EP antibodies, the different binding affinities indicate that the hybridomas derive from independent cell precursors.

Characterization of Monoclonal Antibodies. The antibodies produced by two clones, B10 and B7, isolated from culture 3 were used for further studies. They are indistinguishable on the basis of specificity or structural features—i.e., they were derived from the same progenitor cell hybrid. Both bound to

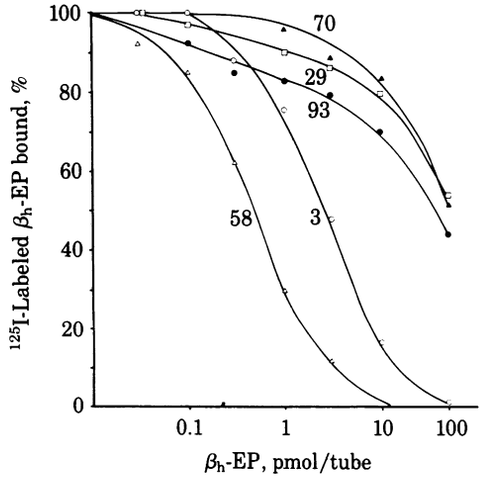

FIG. 1. Displacement of ^{125}I-labeled β-EP from monoclonal antibodies in crude supernatants of five uncloned hybridomas. Bound tracer was separated by double-antibody precipitation. Dilutions of culture supernatants were adjusted to yield tracer binding of about 25% in the absence of unlabeled antigen.

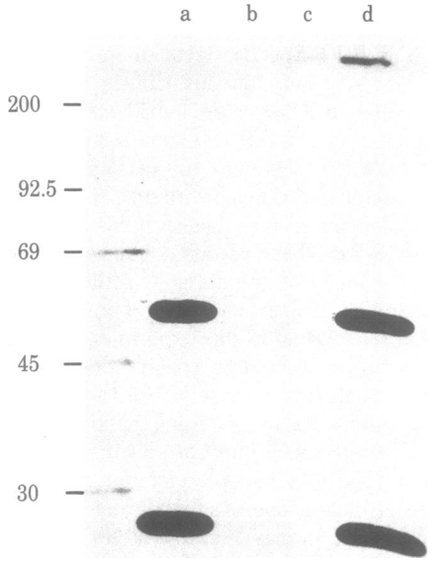

FIG. 2. Autofluorogram of ^{3}H-labeled immunoglobulins from supernatants of cloned hybrids electrophoresed in a $NaDodSO_4$/10% polyacrylamide slab gel. Proteins were adsorbed to protein A-Sepharose beads before direct elution in reducing sample buffer. Radioactive M_r standards were from Amersham (CFA.626). Lanes: a and d, supernatants from clones E7 and B10 derived from original culture 3; b, supernatant from the myeloma parent; c, supernatant from a sister clone that had lost binding activity.

staphylococcal protein A-Sepharose columns and eluted at pH 4.5. The structural similarities and the biochemical homogeneity of the antibody chains produced by the two cell clones were confirmed by $NaDodSO_4$/polyacrylamide gel electrophoresis (Fig. 2). In addition, unpurified supernatants from both clones were shown by enzyme immunoassay to contain only heavy chains of IgG2a type and κ light chains. The clones were stable with respect to antibody production and produced tumors in syngeneic pristane-primed (24) animals. Ascites fluids from tumor-bearing mice contained anti-β-EP antibodies as high as 30 mg/ml. The characteristic binding affinity for protein A-Sepharose has been exploited for a single-step purification of large amounts of antibodies from cell culture medium or ascites fluid.

Crossreactivity Studies. In order to determine the spectrum of crossreactivity of the antibody 3-E7 and possibly to map the corresponding antigenic site on β_h-EP, various opioid peptides were tested for competition with the homologous antigen. Table 1 summarizes the results of several displacement radioimmunoassays. In contrast to all conventional antisera raised against β-EP, antibody 3-E7 failed to recognize β-LPH. Substantial deletions at the COOH terminus of β-EP did not alter the immunoreactivity of the molecule whereas removal or acetylation of the NH_2-terminal residue abolished antigenicity.

The results establish unambiguously that the combining site of antibody 3-E7 is complementary to the enkephalin sequence of β-EP and that the NH_2-terminal tyrosine makes an essential contribution to the binding specificity. Interestingly, the sequence of camel β-EP is identical to that of mouse β-EP. Thus one would expect that this mouse monoclonal antibody would recognize mouse β-EP equally well. We have applied antibody 3-E7 in a radioimmunoassay for the measurement of the ratio of acetylated to nonacetylated β-EP in pituitary extracts from mice (26.3 pmol/mg, 9.3% of total β-EP immunoreactivity) and rats (13.7 pmol/mg, 17.8% of total β-EP immunoreactivity). The data are in agreement with the results of Weber *et al.* (25).

β-EP Monoclonal Antibody Applied to Immunohistochemical Analysis. The use of antibody 3-E7 as an analytical probe for the localization of the natural ligands on tissue sections was explored with the Sternberger peroxidase-antiperoxidase technique and by immunofluorescence. All cells in the intermediate lobe of rat pituitary were intensely stained (Fig. 3). Higher magnification revealed heavy cytoplasmic localization of the immunoreactive peptides. In the anterior lobe, immunoreactivity was observed only within certain disseminated cells, possibly identical to the ones reported to elaborate corticotropin (26). Blocking studies with several opioid peptides (see legend to Fig. 3) confirmed the crossreactivity data obtained for antibody 3-E7 in radioimmunoassay. In sharp contrast to the finding in the anterior pituitary and pars intermedia, the posterior lobe showed no detectable immunoreactivity.

These findings confirm previous immunofluorescence data (27) obtained with conventional antisera which generally also recognize β-LPH (reviewed in ref. 28). However, the total lack of antigenic material in the pars nervosa is somewhat unexpected with antibody 3-E7 in view of its crossreactivity with other related peptides, such as [Met]- or [Leu]enkephalin as well as dynorphin, which have been localized in the posterior lobe of the rat pituitary (29, 30). We note that the pituitary sections had not been treated with detergents, but we have not explored possible technical reasons for this unexpected result by examining different fixation conditions or other handling procedures.

Immunofluorescence labeling of serial sections throughout

Table 1. Crossreactivities of monoclonal antibody 3-E7

Peptide	Relative activity	Sequence
		1 60 61 69 91
β_h-LPH	<0.1	—//—Arg-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro—//—Tyr-Lys-Lys-Gly-Glu
β_h-EP	100	——//——————
β_c-EP	100	——//—His————Gln
β_h-EP$_{61-69}$	100	———————————————
[Met]Enkephalin	80	———————
[Leu]Enkephalin	80	———————Leu
β_h-LPH$_{60-65}$	90	———————————
des-Leu, Met-Enkephalin	5	————————
Acetyl-β_h-EP	<0.1	MeCo———
des-Tyr$_{61}$-β-EP	<0.1	———
β_h-LPH$_{66-91}$	<0.1	———
Dynorphin	16	———————Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys
α-neo-EP	20	———————Leu-Arg-Lys-Tyr-Pro-Lys

Immunoreactivities are expressed relative to the 50% inhibitory concentration of the homologous antigen β_h-EP (10 pmol per tube).

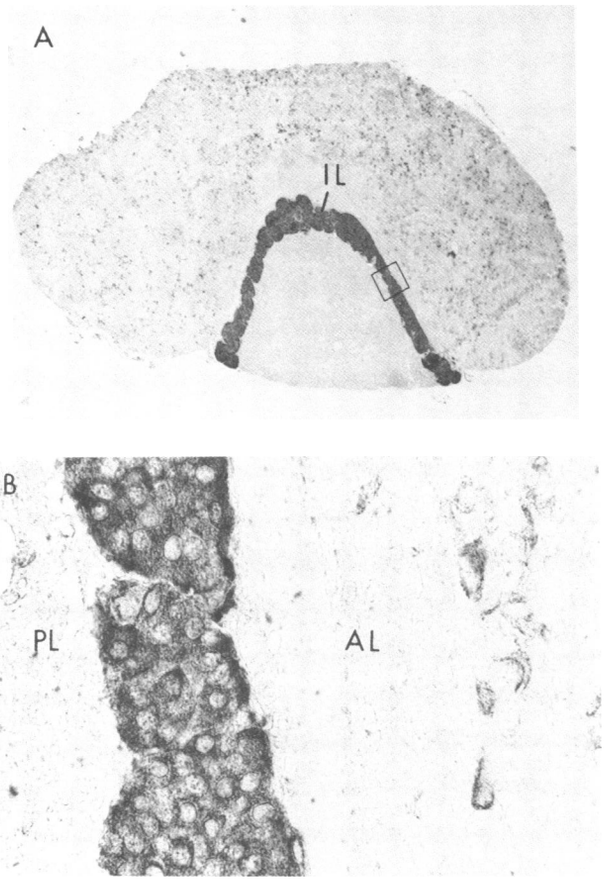

FIG. 3. Peroxidase-antiperoxidase immunostaining of rat pituitary gland with monoclonal antibody 3-E7. (*A*) Low-power photomicrograph showing the intense limited localization of immunoreactive cells in the pars intermedia (IL), visible also by naked eye. (×30.) (*B*) Higher magnification (×320) of the field outlined in *A*, showing the presence of immunoreactive peptides also in the cytoplasm of disperse adenohypophyseal cells (AL) but not in the posterior lobe (PL). Immunostaining was blocked by β_h-EP (20 μM), [Met]enkephalin (50 μM), dynorphin (100 μM), β-neo-EP (100 μM), and [Leu]enkephalin (50 μM) but not by acetylated-β_h-EP (30 μM).

the rat forebrain revealed that the monoclonal antibody detected the neuronal systems that have been reported to react with conventional antisera against β-EP (31) or the enkephalins (32). In addition, the antibody also labeled neurons (Fig. 4) which recently have been shown to react with antisera to dynorphin

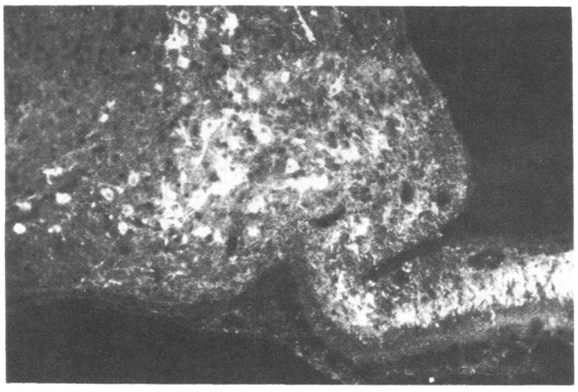

FIG. 4. Immunofluorescence staining of β-EP cell bodies in the arcuate nucleus of rat hypothalamus by monoclonal antibody 3-E7. Bundles of fibers in the internal eminence are also stained. The material in these fibers is dynorphin and α-neo-EP (23). (×120.)

and α-neo-EP (23, 33). These preliminary studies show that antibody 3-E7 allows a clear visualization of antigens in tissue sections and confirm that this monoclonal antibody universally labels neurons containing peptides with the characteristic Tyr-Gly-Gly-Phe at the NH_2 terminus.

DISCUSSION

A common drawback of conventional antisera is that their spectrum of crossreactivity applies only under the assay conditions in which it is defined. Thus, identification of the antigenic determinants recognized by a particular reagent in a radioimmunoassay is not a sufficient safeguard for the interpretation of data obtained in another assay—e.g., immunochemistry or immunoadsorption—in which concentration effects allow for the interference of other unsuspected antibody populations. In contrast, for a monoclonal antibody the capacities of a series of overlapping peptides to inhibit binding to the homologous antigen can establish unequivocally its single combining-site specificity. The antigenic determinant recognized by the anti-β-EP 3-E7 can be finely mapped to the NH_2 terminus of the polypeptide (Table 1). The monoclonal antibody does not discriminate between [Leu]enkephalin and [Met]enkephalin, and it shows strong reactivity with both. Removal of Tyr^{61} from [Met]-enkephalin or β-EP, or of larger peptides from the NH_2 terminus of β-EP, drastically abrogates antigenic activity.

The shortest peptide still able to interact with the combining site of the monoclonal antibody has the sequence Tyr-Gly-Gly-Phe. An additional COOH-terminal residue does not appear to make a substantial contribution to the specificity of the antigenic determinant in that [Met]enkephalin and [Leu]enkephalin are indistinguishable. However, the stronger immunoreactivity of the latter peptides suggests that the added hydrophobic residue contributes an important auxiliary force for the binding of the tetrapeptide to the antibody combining site. Strikingly, all mammalian opioid peptides have this tetrapeptide as the canonical sequence responsible for their pharmacological activity. Thus, it would appear that the combining site of the monoclonal antibody 3-E7 shares some stereochemical specificity with the opioid receptor itself.

A number of studies utilizing omission, replacement, or truncated analogs of β-EP-related peptides have established the structural requirements for the binding and biological activity of the opioid peptides (34–37). It has emerged that multiple structural features determine ultimate biological activity by influencing susceptibility to degradation, receptor selectivity, and potency. Analysis of the last two features strongly indicates the existence of several classes of opioid receptors with distinct anatomical distribution (38). Although the biochemical relationships among the various receptors remain unclear (35), they share a site complementary to the sequence Tyr-Gly-Gly-Phe, designated "message sequence" (39).

The series of β-EP-related polypeptides we have analyzed for immunoreactivity with the monoclonal antibody cover only a few of the modifications known to affect the binding of the message sequence to the opioid receptors. Nonetheless, the ones tested show an astonishing correlation in the abrogation of immunoreactivity and receptor-binding activity. The primary importance of the presence and configuration of the NH_2-terminal tyrosine also for antibody binding is shown by the lack of immunoreactivity of the des-Tyr^1 derivative and the acetylated form of β-EP. Likewise, the extent of iodination of Tyr^1 also affects immunoreactivity [in contrast to iodination of Tyr^{27} (results not shown)]. On the other hand, the NH_2-terminal addition of an arginyl residue (β-LPH_{60-65}) appears to be equally tolerated in our antibody binding assay as well as in receptor

binding assays (36). More strikingly, the tetrapeptide sequence able to retain detectable immunoreactivity with 3-E7 is the same minimal fragment that shows definite affinity to brain opiate receptors (40) and activity in the guinea pig ileum bioassay (41). Finally, the tertiary structure of the precursor β-LPH hinders the interaction of its internal message sequence with the opioid receptor as well as with the combining site of the monoclonal antibody.

Conventional antisera against β-EP have generally shown a lack of correlation between antigenicity and opiate or other biological activities (e.g., ref. 37). In contrast, the observations reported here indicate that monoclonal antibody 3-E7 has the prerequisites of an universal ligand for the naturally occurring and biologically active mammalian opioid peptides. It is noteworthy that the sequence of mouse β-EP deduced from a β-LPH cDNA clone (42) differs from the human one only in two COOH-terminal residues, His^{27} and Gln^{31}. If β-EP and [Met]-enkephalin circulate free in mouse plasma as in human plasma (43, 44), it follows that the antibody we have described is a monoclonal autoantibody. Mouse autoantibodies have been already immortalized in myeloma cell hybrids (45). The failure of this monoclonal antibody to discriminate among opioid peptides carrying the same message sequence is only a virtual limitation. Its specificity in radioimmunoassay can be targeted more finely if the antibody is used in combination with a second antiserum or with one of the other anti-β-EP monoclonal antibodies we have obtained. In fact, the specificity of antibody 3-E7 is a valuable attribute of this reagent because it may lead to the identification of other intermediary forms or novel opioid peptides sharing the canonical sequence Tyr-Gly-Gly-Phe.

The similarity of the specificity of the antibody-combining site and that of the opioid receptors lends itself to the anti-idiotypic antibody approach (46) for obtaining a receptor-specific probe.

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