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MONOCLONAL ANTIBODY TO ENKEPHALINS WITH BINDING CHARACTERISTICS SIMILAR TO OPIATE RECEPTOR

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Summary: Monoclonal antibodies to enkephalins were established by immunization of mice with met-enkephalin, leu-enkephalin or both. Twenty-three clones with a high titer were classified into 6 types according to the binding properties to enkephalins and their derivatives. Antibody LM 239 showed binding characteristics similar to opiate receptor. It has a very high affinity to enkephalins and their derivatives which have a potent opioid activity, but a low affinity to enkephalin derivatives which devoid of opioid activity. The binding of ^{3}H-met-enkephalin to the antibody was inhibited by naloxone and morphine, although the ID_{50} values were considerably higher than the Ka values of the alkaloids to opiate receptor. © 1985 Academic Press, Inc.

Immune system produces a variety of immunoglobulins that specifically recognize foreign substances, antigens. Memory B cells contain membrane receptors for antigens with the same specificity as immunoglobulins. By the same token, membrane receptors recognize transmitters or hormones from extracellular origins in a specific manner and transduce their signals into intracellular events. The recognition mechanism and the structure of receptor molecules might bear some resemblance to immunoglobulins. Recently several reports appeared showing that the antibodies raised against neurotransmitters or hormones exhibited binding specificities comparable to membrane receptors (1-5). However, most of these studies were carried out with antisera, which made it difficult to compare the detailed properties of antibodies and receptors.

Several laboratories have produced antisera to opioid peptides including enkephalins (6-8). The binding characteristics of the antisera differed considerably from each other. None of the antisera so far reported exhibited binding characteristics that mimicked opiate receptor. We employed a

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monoclonal hybridroma technique (9) to produce antibodies to enkephalins and compared their properties. Here we report the production and properties of the monoclonal antibody with binding properties similar to opiate receptor.

EXPERIMENTAL PROCEDURES

Chemicals: [Tyrosyl-3,-5-^{3}H]5-L-leucine enkephalin (43 Ci/mmol) and [tyrosyl-3,5-^{3}H]5-L-methionine enkephalin (51 Ci/mmol) were purchased from Amersham, UK, and [tyrosyl-3,5-^{3}H]2-D-alanine^2-5-L-methionine enkephalinamide (43.6 Ci/mmol) from New England Nuclear, Boston, Mass. Enkephalins and their derivatives were obtained from Protein Research Foundation, Osaka, Japan, or Sigma Chemical Company, St Louis, MO.

Immunization of mice: Met-enkephalin and leu-enkephalin were coupled to BSA with 1-ethyl-3(3-dimethylaminopropyl)carbodiimide·HCl as described (7). A BALB/c mouse was injected intraperitoneally with 350 µg enkephalin-BSA with 2 X 10^9 heat-inactivated pertussis vaccine. Three weeks later, the animals received a booster (350 µg enkephalin-BSA) without pertussis vaccine. Three days after the booster injection, spleen cells (6-9 X 10^8) were fused with mouse myeloma cells SP-2/0 Ag-14 (1 X 10^8) by the addition of 2.5 ml of 50% polyethylene glycol 4000. There were 4 mice, a mouse in each group, with different immunization schedules: LL mouse received leu-enkephelin-BSA for both priming and booster; MM mouse received met-enkephalin-BSA for both priming and booster; LM mouse was injected with leu-enkephalin-BSA for priming and met-enkephalin-BSA for booster; ML mouse was injected with met-enkephalin-BSA for priming and leu-enkephalin-BSA for booster. Fused cells were suspended in HAT selection medium (RPMI medium supplemented with 15% fetal calf serum, 2 mM L-glutamine, 1 mM pyruvate, 100 µM hypoxanthine, 0.4 µM aminopterine and 16 µM thymidine), and inoculated into 96-well Falcon Microtest plates (0.1 ml per well) at a concentration of 1 X 10^6 cells per well. Four days later, 0.1 ml of HT medium (HAT medium without aminopterine) was added. Culture fluids showing hybrid growth were tested for the production of antibody to enkephalins.

Screening of antibody: Culture fluid (0.1 ml) was mixed with 0.05 ml of reaction mixture containing 12.5 µmol of Tris-HCl (pH 7.6), 125 µg of bovine IgG and 0.5 pmol of ^{3}H-met-enkephalin or ^{3}H-leu-enkephalin. After incubation in ice for 1 h, 0.15 ml of saturated $(NH_4)_2SO_4$ solution was added to the reactions followed by centrifugation at 1500 x g for 20 min. The precipitates were dissolved in 0.25 ml of distilled water and the radioactivity was measured by a scintillation counter. The hybridomas positive for enkephalin antibody were cloned by limited dilution method.

Purification of antibody: Ascites fluid was obtained from Pristan-primed BALB/c mice injected with 1 X 10^7 hybridoma cells and purified by a protein A Sepharose column (1 X 7 cm) chromatography (10).

Characterization of binding properties: The reaction mixture contained 25 µmol of Tris-HCl (pH 7.6), 150 µg of bovine IgG, 0.4 pmol of ^{3}H-met-enkephalin and purified antibody (20-30 ng) in a total volume of 0.5 ml. Non-radioactive enkephalins or their derivatives to be tested were included as indicated. After incubation in ice for 1 h, solid $(NH_4)_2SO_4$ was added to 50% saturation and centrifuged at 1500 X g for 20 min. The radioactivity in precipitate was measured as described above. As blanks, reactions without antibody were run and the values were subtracted from those with antibody.

RESULTS

Culture fluids from approximately 2700 wells containing growing hybridoma cells were tested for the binding activity to ^{3}H-met-enkephalin and ^{3}H-leu-enkephalin. Twenty-three clones with a very high titer to either

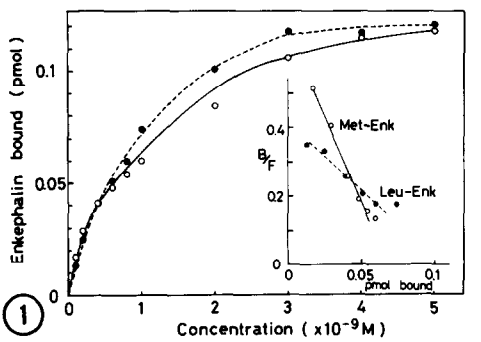
met-enkephalin or leu-enkephalin or both were established after limited dilution method. The binding properties of the antibodies were qualitatively characterized using culture fluids and the antibodies were classified into 6 types (Table I). Type I and II showed a very high affinity to met-enkephalin, leu-enkephalin, D-alanine^2-L-met-enkephalinamide, and α-endorphin. Type III and IV had a very high affinity to both met-enkephalin and leu-enkephalin, but no or a low affinity to D-alanine^2-L-met-enkephalinamide. Type V and VI discriminated between met-enkephalin and leu-enkephalin showing a higher affinity to the respective enkephalin employed as antigen.

Among these hybridomas, LM 239, LM 221, LM 345, ML 114, MM 654 and LL 785 cells were injected into mice and the antibodies were purified from ascites fluids. Antibody LM 221, LM 345, ML 114, MM 654 and LL 785 showed almost identical binding characteristics to enkephalins and their derivatives (data not shown) indicating that they produced the same or a very similar antibody. Antibody LM 239 differed from type II in one respect that the binding of ^{3}H-met-enkephalin was inhibited by naloxone and morphine. The detailed

Table I. Binding properties of monoclonal antibodies to enkephalins

Type of antibodies	Met-enkephalin	Leu-enkephalin	Enkephalinamide	α-Endorphin	Naloxone	Clone No
I	+++	+++	+++	+++	+	LM 239
II	+++	+++	+++	+++	-	LM 62, 190, 213, 221 286, 345, 419 ML 114 MM 654, 735 LL 785
III	+++	+++	+	ND	-	MM 433, 444, 615
IV	+++	+++	-	ND	-	LL 92, 202, 213, 329
V	+++	-	-	ND	ND	MM 16, 214
VI	+	+++	-	ND	-	LL 420, 595

Binding of met-enkephalin (L-methionine^5 enkephalin), leu-enkephalin (L-leucine^5 enkephalin) and enkephalinamide (D-alanine^2 L-methinonine^5 enkephalinamide) was measured using 0.5 pmol of ^{3}H-labelled ligands and 10-20 µl of culture fluids as described under EXPERIMENTAL PROCEDURES. Binding of α-endorphin and naloxone was measured by the inhibition of ^{3}H-met-enkephalin binding to the antibodies.
ND, not determined.



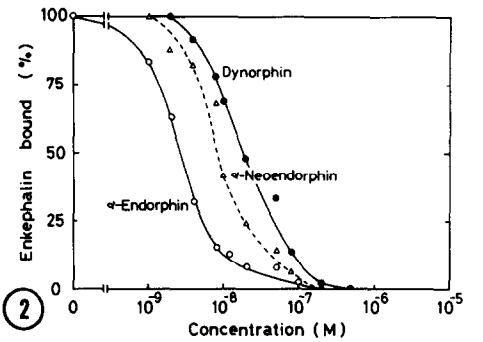


Fig. 1. Saturation binding and Scatchard plots (inset) of the binding of met-enkephalin ○ and leu-enkephalin ● to antibody LM 239. Below 0.8 nM, ^{3}H-labelled ligands were used. Above 1 nM, non-radioactive enkephalins were added to reactions with 0.4 pmol of ^{3}H-ligands. The values are the means of triplicate determinations.

Fig. 2. Effect of α-endorphin ○, α-neoendorphin △ and dynorphin ● on the binding of ^{3}H-met-enkephalin to antibody LM 239. The values are the means of triplicate determinations.

properties of antibody LM 239, identified as IgG_1, are described in this communication.

Antibody LM 239 recognized both met-enkephalin and leu-enkephalin to a very similar degree with the Ka values of 0.3 nM and 0.6 nM for met-enkephalin and leu-enkephalin, opioid peptides first described by Hughes et al (11), respectively (Fig. 1). The antibody also showed a very high affinity to other opioid peptides. The binding of ^{3}H-met-enkephalin to the antibody was effectively inhibited by either α-endorphin, or α-neoendorphin, or dynorphin (Fig. 2), all of which were shown to have a potent opioid activity (12-14). ID_{50} were approximately 5 nM, 9 nM and 30 nM for α-endorphin, α-neoendorphin and dynorphin, respectively. Under the assay condition used here, Ki values were estimated to be approximately one-quarter of ID_{50} values according to the equation proposed by Cheng and Prusoff (15). The antibody also showed a high affinity to D-alanine derivatives of enkephalins, which were shown to be active opioid analogues (Fig. 3)(16,17). ID_{50} of D-alanine^{2}-L-met-enkephalin and D-alanine^{2}-L-met-enkephalinamide were 3 and 4 nM, respectively, while D-alanine^{2}-D-leu-enkephalin was less effective.

Tyr-gly-gly-phe was shown to have an intrinsic opioid activity 2 to 3 orders lower than enkephalins (18). Tyrosine at NH_2 terminal was essential

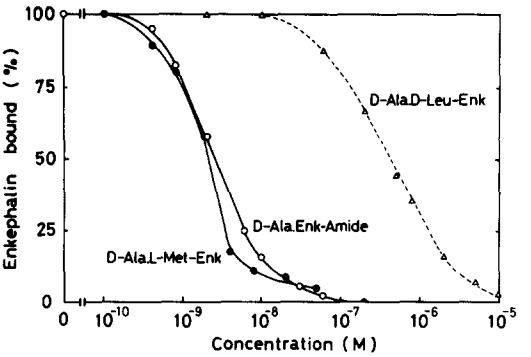
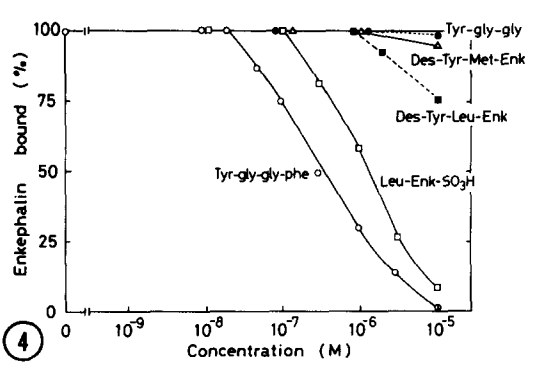


Fig. 3. Inhibition of the binding of ^{3}H-met-enkephalin by D-alanine^{2} L-methionine^{5} enkephalin (D-Ala.L-Met-Enk) ● , D-alanine^{2} L-methionine^{5} enkephalinamide (D-Ala.Enk-Amide) ○ and D-alanine^{2} D-leucine^{5} enkephalin (D-Ala.D-Leu-Enk) △ . The points represent the means of triplicate assays.

for opioid activity. Removal or chemical modifications of tyrosine moiety in enkephalins resulted in a dramatic loss of opioid activity (19,20). In accordance with the potency of opioid activity, tyr-gly-gly-phe, des-tyr-leu-enkephalin, des-tyr-met-enkephalin, and O-sulfated leu-enkephalin did not bind, or slightly bound to the antiobdy (Fig. 4). Tyr-gly-gly did not inhibit the binding of ^{3}H-met-enkephalin to the antibody upto 0.1 mM. Naloxone and morphine, antagonist and agonist of alkaloid compounds, also prevented the binding of ^{3}H-met-enkephalin to the antibody (Fig. 5), although



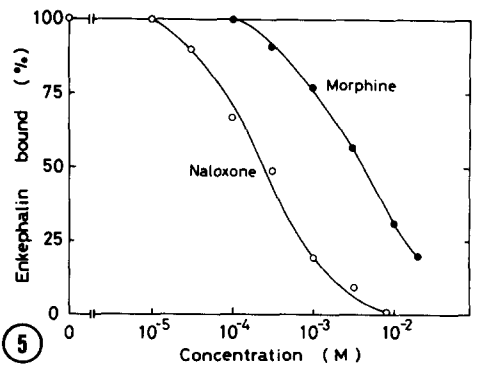


Fig. 4. Inhibition of the binding of ^{3}H-met-enkephalin by tyr-gly-gly-phe ○ , tyr-gly-gly ● , des-tyr-met-enkephalin △ , des-tyr-leu-enkephalin ■ and O-sulfated L-leu-enkephalin (Leu-Enk-SO_3H) □ . The points represent the means of triplicate assays.

Fig. 5. Effect of naloxone ○ and morphine ● on the binding of ^{3}H-met-enkephalin to antibody LM 239. The assays were carried out in triplicates.

ID_{50} was considerably higher than the affinity of the alkaloids to opiate receptor.

DISCUSSION

The present study reports the production and characterization of the monoclonal antibodies to enkephalins after 4 different immunization schedules. Twenty-three clones were classified into 6 types according to their binding properties. The affinities to enkephalins, their derivatives and opioid alkaloids were slightly different between 6 types of antibodies, indicating that the structures of hypervariable regions must slightly differ between 6 types. Among the antibodies established, the binding characteristics of antibody LM 239 were in many respects similar, though not identical, to the binding properties of opiate receptor. It has a very high affinity to enkephalin derivatives which have a potent opioid activity and an apparent affinity to naloxone and morphine, but no or a low affinity to enkephalin derivatives which devoid of opiate activity. The existence of three or four subtypes of opiate receptor, δ, κ, μ and σ, have been proposed from pharmacological studies. The binding characteristics of opiate receptors to opioid compounds differed considerably between subtypes of receptors, and between different animals and different tissues (18,21). Therefore, it is difficult to assign to which subtypes of opiate receptors our antibody resembled. Since δ-receptor has a lower affinity to alkaloids and a higher affinity to enkephalins and their derivatives, antibody LM 236 is most likely to resemble to δ-receptor.

During this study, two laboratories have reported the production of the monoclonal antibodies to enkephalins. Their antibodies, however, have no resemblance to opiate receptors in their binding properties (22,23). Gramsch et al. also reported the production of monoclonal antibodies to β-endorphin which recognized the sequence of tyr-gly-gly-phe exhibiting the specificity requirement of opiate receptor. However, their antibodies cross-reacted with neither D-alanine derivatives nor opioid alkaloids, indicating a distinct property from opiate receptor (24,25).

Recently several laboratories have reported that antibodies raised against hormones or neurotransmitters such as insulin, chemotactic peptide, l-alprenolol and BisQ, showed binding affinities similar to their respective receptors (1-5). The anti-idiotype antibodies also recognized both idiotype antibodies and respective receptors (1,4,5,26-28). However, these studies were carried out with antisera, which presumably contained a large number of antibodies with different specificities. Monoclonal antibody would offer a procedure to explicitly analyze the similarity and difference between idiotype antibodies and receptors. Recently Cleveland et al. produced monoclonal antibodies to BisQ, a nicotinic agonist, with binding properties similar to nicotinic receptor. They further obtained a monoclonal anti-idiotype antibody which cross-reacted with actylcholine receptors from Torpedo Californica and rat muscle (29). We have also established a monoclonal antibody against choline-hemiglutarate-BSA, which exhibited binding affinities similar to nicotinic receptor (Nakane, M. and Deguchi, T. in preparation). Thus among a variety of antibodies, some antibodies could have close resemblance to the receptors for transmitters or hormones which were employed as antigens. It might be possible that the structures of antibodies and receptors to recognize the ligands derived from the same ancestral genes. Production of the anti-idiotype antibodies with a high specificity to receptors would offer a good tool to analyze the properties and structures of receptors evading the difficulties encountered in the purification of minute amount of receptor proteins. We are presently trying to produce monoclonal anti-idiotype antibodies to antibody LM 239.

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