BIBLID: ISSN 0253-9756 Acta Pharmacologica Sinica 中国药理学报 1994 Jul; 15 (4): 303—307

Monoclonal antibodies specific for ohmefentanyl[1]

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ABSTRACT Ohmefentanyl (Ohm, *N*[1-(β-hydroxy-β-phenylethyl)-3-methyl-4-piperidyl]-*N*-phenylpro-pronamide), designed and synthesized by our laboratory, is a highly selective μ receptor agonist. After somatic cell fusion between splenocytes of BALB c mice, immunized by Ohm-BSA conjugate and NS-1 myeloma cells, 2 lines of hybridoma (D_2 and F_4) secreting monoclonal anti-Ohm antibodies (MAb) were obtained. Saturation and competition experiments showed that MAb-D_2 and MAb-F_4 bound to Ohm-BSA with high affinity and high specificity. Using radioligand binding assay and bioassay, we also found that MAb-D_2 and MAb-F_4 inhibited [^{3}H] Ohm binding to rat brain opioid receptors in a dose-dependent manner and antagonized the effect of Ohm on the contraction of guinea pig ileum induced by electric field stimulation. These results suggested that MAb-D_2 and MAb-F_4 were 2 monoclonal antibodies specific for Ohm and could be useful as functional antagonists of Ohm.

KEY WORDS ohmefentanyl; mu receptors; monoclonal antibodies

Ohmefentanyl (Ohm, *N*[1-(β-hydroxy-β-phenylethyl)-3-methyl-4-piperidyl]-*N*-phenylpro-pronamide), designed and synthesized by our laboratory, is a highly selective μ receptor agonist[1-3]. At present, we wish to produce monoclonal antibodies oriented to Ohm, as a possible immunogen for the production of anti-Ohm anti-idiotypic antibodies which may carry an internal image of Ohm and may be useful in the isolation and identification of μ receptor binding protein.

Received 1993-10-23 Accepted 1994-03-15

[1] Project supported by the National Natural Science Foundation of China, № 938900706.

MATERIALS AND METHODS

1 Animals and drugs BALB c ♀ mice weighing 20±*s* 2 g (Shanghai Institute of Cell Biology, Chinese Academy of Sciences). Sprague-Dawley rats ♂ weighing 250±*s* 30 g and guinea pigs ♂ weighing 380 ±*s* 50 g (Shanghai Institute of Materia Medica, Chinese Academy of Sciences). The myeloma cell line NS-1 (Cell Banks of Shanghai, Chinese Academy of Sciences).

Ohm, β-hydroxylfentanyl, 3-methylfentanyl, fentanyl, [*D*-Ala^2, *D*-Leu^5]enkephalin (DADLE), and etorphine (Shanghai Institute of Materia Medica). Morphine (Qinhai Pharmaceutical Factory). Naloxone (Shanghai Medical University). Dihydroetorphine (Chinese Academy of Military Medical Sciences). [*D*-Ala^2, Mephe^4, Glyol^5]enkephalin (DAGO), [*D*-Pen^2, *D*-Pen^5]enkephalin (DPDPE), and *N*-methyl-*N*-[7-(1-pyrrodidinyl)-1-oxaspiro(4,5)dec-8-yl]benzeneacetamide (U-69593) (Sigma Co). [^{3}H]Ohm (2.02 PBq·mol^{-1}) (Shanghai Institute of Nuclear Research, Chinese Academy of Sciences).

2 Production of monoclonal anti-Ohm antibodies (MAb)

Immunization Ohm-6-hemisuccinate was prepared and conjugated to bovine serum albumin (BSA) (molar ratio, 47:1). BALB c mice were injected intrasplenically (20 μg of Ohm-BSA) and ip (100 μg of Ohm-BSA) alternatively at 3—4 wk intervals. Sera were tested periodically for anti-Ohm antibodies by radioimmunoprecipitation test and enzyme-linked immunosorbent assay (ELISA). By the end of immunization period, 100 μg of the Ohm-BSA was injected

both ip and iv. After 3 d the mouse splenocytes were harvested for cell fusion.

Fusion About 1×10^8 splenocytes were fused with 5×10^7 myeloma cells in the presence of 0.7 ml 40 % polyethylene glycol 4000, washed in Dulbecco's modified Eagle medium (DMEM), and resuspended in DMEM-fetal calf serum. On d 1, 3, 6, and 10, a fresh medium containing hypoxanthine, aminopterin, and thymidine (HAT) was added. After d 15, the culture supernatants were tested for anti-Ohm antibodies by ELISA.

Subcloning of positive hybridoma Hybridoma cell populations secreting anti-Ohm antibodies were subcloned for 4—5 times to obtain the monoclonal cell line by limiting the dilution on feeder cell layers in microtiter polystyrene plates.

Amplification in the ascites Monoclonal hybridoma cell lines were incubated in Petri dishes and 2×10^6 cells of each line were injected ip into mice previously primed with 0.5 ml of pristane. Ascitic fluid was centrifugated at $110 \times g$ to remove cells.

3 Purification of MAb The anti-Ohm MAb were purified from ascitic fluids by 2-step precipitation using 50 % and 33 % saturation of $(NH_4)_2SO_4$, respectively or by protein A — Sepharose column chromatography. MAb were assessed by SDS-PAGE and immunoprecipitation to identify the purity and Ig subgroups.

4 Radioimmunoprecipitation test [^{3}H] Ohm (0.1 ml of 2 nmol·L^{-1}), serial dilutions of antiserum (0.05 ml), and Tris-HCl buffer (0.35 ml of 50 mmol·L^{-1}) were mixed and incubated at 4 ℃ for 16 h. Rabbit anti-mouse IgG antibodies (0.03 ml) and 1 % normal rabbit serum (0.03 ml) were added at 4 ℃ for 4 h. [^{3}H]Ohm-antibody compounds were isolated by centrifugation and counted by a liquid scinitillation counter (YSJ-80, Shanghai Institute of Nuclear Research).

5 ELISA Ohm-BSA (0.1 ml of 50 μg·ml^{-1}) was coated on immunoplates. After washing and blocking with PBS containing 10 % bovine serum, 0.1 ml of anti-Ohm MAb at different concentrations were added and incubated 2 h at 37 ℃. After washing, 0.1 ml of sheep anti-mouse IgG antibodies coupled with peroxidase were mixted for 2 h further. Peroxidase activity was revealed by 0.04 % H_2O_2-OPD substrate. A_{490} was measured by an automatic microplate reader (BIO-TEK EL311).

6 Competitive ELISA Serial dilutions of opioid ligands (0.1 ml) and anti-Ohm MAb (0.1 ml) were mixed before adding to the well-plates previously coated with Ohm-BSA 50 μg·ml^{-1}, and then incubated for 2 h at 37 ℃. Other conditions were specified for the normal ELISA.

7 Radioligand binding assay Rat brain membranes were prepared[17]. [^{3}H]Ohm (0.1 ml of 2 nmol·L^{-1}), rat brain membranes (0.3 ml, 1 mg protein), and increasing concentrations of anti-Ohm MAb (0.1 ml) were mixed at 30 ℃ for 45 min. Nonspecific binding was measured in the presence of 0.1 ml of unlabeled Ohm 1 μmol·L^{-1}. Binding was terminated by rapid filtration and quantitated using a liquid scintillation counter.

8 Bioassay The segment of guinea pig ileum was prepared[23]. The contraction of ileum was induced by electric field stimulation (40 V, 1 ms, 15 s intervals). Electric field stimulating contraction (ESC) under such conditions could be inhibited by Ohm, and the effects of anti-Ohm MAb on inhibition of Ohm were observed.

RESULTS

1 Detection of anti-Ohm antisera After repeatedly immunizing the BALB c mice with Ohm-BSA for more than 10 times, the titers of anti-Ohm antisera lay between 1∶1600 and 1∶3200 at 30 % of the ratio of [^{3}H]Ohm-antibody to [^{3}H]Ohm (Fig 1).

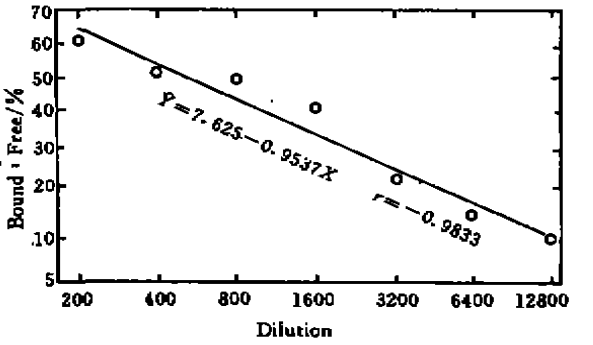

Fig 1. Titers of mouse anti-Ohm antiserum detected by radio-immunoprecipitation test. Each point is mean of three independent experiments.

Preincubated the antisera with 10 % bovine serum at 37 C for 2 h, the titers of anti-Ohm antisera by ELISA was assayed. The titers lay between 1 : 2000 and 1 : 4000 while A_{490} was 1.00, which was identical with the titers detected by radioimmunoprecipitation test (Fig 2).

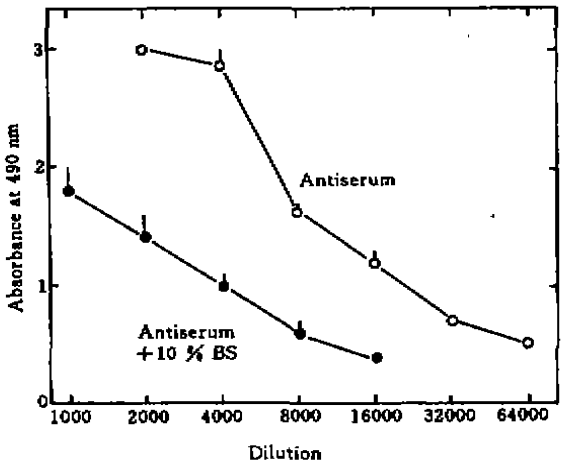

Fig 2. Titers of mouse anti-Ohm antiserum detected by ELISA. $n=4$, $\bar{x}\pm s$.

2 Identification of immunoglobulins

After cell fusion, 2 lines of hybridomas (D_2 and F_4) secreting monoclonal anti-Ohm antibodies were obtained. Immunoprecipitation study showed that the purified immunoglobulins Ig of MAb-D_2 and MAb-F_4 were all of the IgG class. Further analysis showed that the subclasses of MAb-D_2 and MAb-F_4 belonged to IgG_1.

Electrophoresis was performed using SDS-15 % PAGE in the presence of 1 % mercaptoethanol. The 2 bands with molecular weights of 56 300 and 24 200 were shown to be of heavy and light chain, respectively (Fig 3).

3 Binding of MAb to antigen

Anti-Ohm MAb binded to Ohm-BSA in a saturation and dose-dependent manner. The A_{50} of

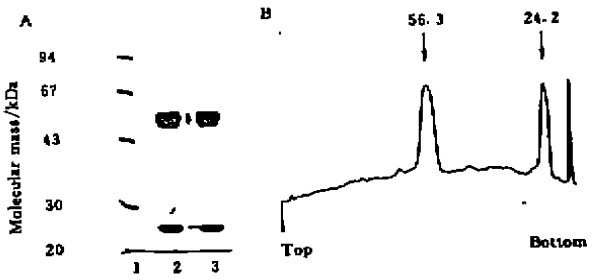

Fig 3. A. SDS-PAGE of monoclonal anti-Ohm antibodies. 1) Markers of indicated molecular mass. 2) MAb-D_2 and 3) MAb-F_4 purified by 2-step precipitation using 50 % and 33 % $(NH_4)_2SO_4$ saturation. B. Scanning graph of lane 2.

MAb-D_2 and MAb-F were 1.1±0.3 and 1.3 ±0.7 nmol·L^{-1} ($n=5$, $\bar{x}\pm s$), respectively. In the same experiments, MAb did not bind to BSA and BSA nor inhibited the binding of MAb to Ohm-BSA (Fig 4).

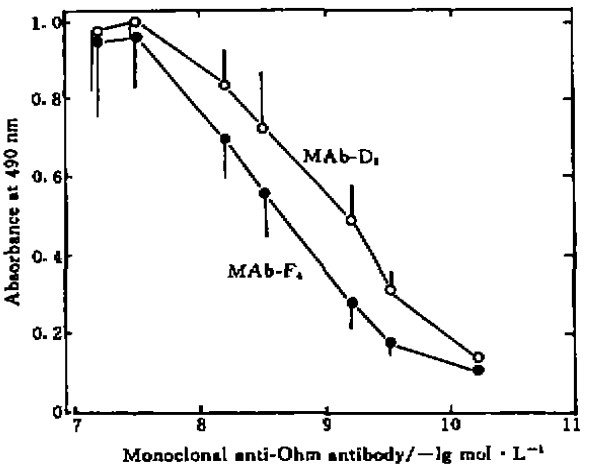

Fig 4. Saturation of MAb binding to Ohm-BSA. $n=5$, $\bar{x}\pm s$.

4 Cross reactivity The cross reactivity of anti-Ohm MAb with a number of selected opioid agonists and antagonists were studied using competitive ELISA. Results showed that Ohm and its analogs inhibited the binding of MAb to Ohm-BSA in a dose-dependent manner, but DAGO, morphine, etorphine, DPDPE, DADLE, and U-69593 showed no

effect. Although dihydroetorphine and naloxone slightly inhibited the binding of MAb to Ohm-BSA in concentrations of >10 $\mu mol \cdot L^{-1}$, the rates of cross reactivity were <1.0 % (Tab 1).

Tab 1. Cross reactivity of MAb-D_2 and MAb-F_4 with opioid ligands. $n=3-6$, $\bar{x}\pm s$.

Ligands	IC_{50} ($\mu mol \cdot L^{-1}$)		Rate of cross reactivity (%)	
	D_2	F_4	D_2	F_4
Ohmefentanyl	0.8±0.5	0.5±0.3	100	100
β-Hydroxylfentanyl	14±4	12±6	5.7	4.2
3-Methylfentanyl	21±3	18±13	3.7	2.7
Fentanyl	53±11	64±30	1.5	<1.0
Dihydroetorphine	>100	>100	<1.0	<1.0
Naloxone	>100	>100	<1.0	<1.0

5 Radioligand binding assay Anti-Ohm MAb inhibited the specific binding of [^{3}H]Ohm to rat brain opioid receptors in a dose-dependent manner. IC_{50} values of MAb-D_2 and MAb-F_4 were 0.18±0.09 and 0.45±0.10 $\mu mol \cdot L^{-1}$ ($n=5$, $\bar{x}\pm s$), respectively (Fig 5).

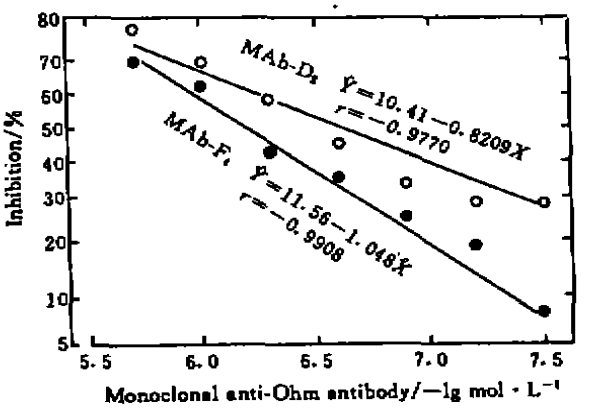

Fig 5. Inhibition of [^{3}H]Ohm binding to rat brain opioid receptors by MAb. Each point is mean of five independent experiments.

6 Bioassay Although anti-Ohm MAb did not influence the ESC of guinea pig ileum directly, it antagonized the inhibition of Ohm on the ESC of guinea pig ileum. MAb-D_2 and MAb-F_4 (1 $\mu mol \cdot L^{-1}$ each) respectively antagonized 81±9 and 61±13 % ($n=4$, $\bar{x}\pm s$) of Ohm (0.25 $nmol \cdot L^{-1}$) inhibition on the ESC (Fig 6).

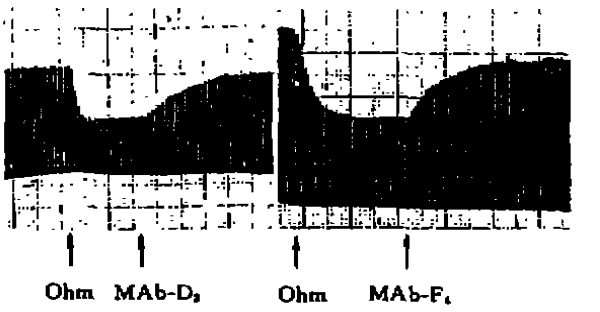

Fig 6. MAb (1 $\mu mol \cdot L^{-1}$) antagonized the effect of Ohm (0.25 $nmol \cdot L^{-1}$) on electric field stimulating contraction of guinea pig ileum.

DISCUSSION

Because of its size, Ohm may not be immunogenic on itself. It should be coupled to a carrier protein before immunization. In our study, Ohm-6-hemisuccinate was prepared and was conjugated to BSA. During immunization with Ohm-BSA, however, antibodies to BSA will also be produced. By simple reasoning, 2 antigens with different carriers should be used, one for immunization, the other for screening of hybridomas secreting anti-hapten antibodies. Ji and Xu reported that using one antigen, they also detected the anti-hapten antibodies in screening of lymphocyte hybridomas [4]. According to their method modified by us, we also detected anti-Ohm antibodies in screening of the hybridomas by blocking the anti-BSA antibodies with 10 % bovine serum by ELISA.

In preliminary studies, the titers of anti-Ohm antisera of mice, rat, and rabbit immunized with conventional methods were very low (<1:100). Opioid agonists (eg Ohm)

have immunosuppressive effect[5,6]. Hence, we chose intrasplenic injection for immunization, an effective method for low immunocompetenr antigen[7], and finally a high-titer anti-Ohm anrtserum in BALB c mice was obtained.

Saturation and competition experiments showed that MAb-D_2 and MAb-F_1 were 2 high affinity and highly specific monoclonal anri-bodies against Ohm. The relative potencies of Ohm analogs inhibiting the binding of MAb to Ohm-BSA were in the order of Ohm > β-hydroxylfentanyl > 3-methylfentanyl > fentanyl. This closely paralleled that their binding capabilities to the opioid receptors[8].

MAb, interacted with Ohm, inhibited the specific binding of [^{3}H]Ohm to rat brain opioid receprors and antagonized the effect of Ohm on guinea pig ilium. Ohm was a highly selective ligand of μ receptors. Anti-Ohm MAb, funcrioned as antagonisrs, may be useful in studying the effect and mechanism of Ohm on the μ receptors.

Anti-Ohm MAb were also used in the anri-Ohm anti-idiorypic antibodies (anti-Id) inducing. According to the nerwork rheory of the immune system[9], among the anti-Ohm anti-Id certain anri-Id bearing the "internal image" of Ohm would be identified. These anti-Id antibodies have brighr prospecr in the research of opioid receptors such as in the isolation of μ receptor binding protein, screening of cDNA clone encoding μ receptors, and so on.

ACKNOWLEDGMENT Ms HONG Long-Sheng taught us the monoclonal antibody techniques.

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抗羟甲芬太尼单克隆抗体

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摘要 采用细胞融合和单克隆技术，获得抗羟甲芬太尼(ohmefenranyl, Ohm)单克隆抗体 MAb-D_2和 MAb-F_1。MAb-D_2和 MAb-F_1与 Ohm-BSA 结合亲和力高、专一性强。MAb-D_2和 MAb-F_1还能抑制[^{3}H]Ohm 与大鼠脑匀浆膜蛋白上的阿片受体的结合，并能对抗 Ohm 抑制电场刺激引起豚鼠回肠收缩的作用。以上结果表明 MAb-D_2和 MAb-F 是特异性的抗 Ohm 单克隆抗体，作为 Ohm 的拮抗剂具有广泛用途。

关键词 羟甲芬太尼；μ 受体；单克隆抗体