Preparation of monoclonal antibody against human m_3 receptor[1]

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KEY WORDS muscarinic receptors; monoclonal antibodies; enzyme-linked immunosorbent assay; radioligand assay; immunohistochemistry

ABSTRACT

AIM: To raise monoclonal antibody against human m_3 receptor. **METHODS**: The m_3 receptor selective peptide segments deduced from its gene were chemically synthesized, coupled to keyhole limpet hemocyanin carrier protein, and injected to Balb/c mice to raise monoclonal antibody. Antibody was purified by a combination of two-step precipitation methods and ion-exchange chromatography. The specificity of monoclonal antibody was tested by enzyme-linked immunosorbent assay, immunohistochemistry, and radioligand binding assay of receptors. **RESULTS**: The monoclonal antibody specifically bound to the protein of rat salivary gland and m_3 peptide, but not m_4 peptide. In radioligand binding assay of receptors, monoclonal antibody inhibited the binding of ^{3}H-QNB to muscarinic receptor in rat salivary gland, but not in rat heart, and could not inhibit the binding of ^{3}H-PZ to rat brain cerebral cortex membrane protein. Immunohistochemical study showed that the human salivary cell surface was strongly stained, whereas the human heart cell surface was not. **CONCLUSION**: Highly purified (96.3 %) monoclonal antibody against the m_3 receptor peptide recognized the m_3 receptor.

INTRODUCTION

Cloning studies confirm the existence of at least five subtypes of mAChR, each one with its own gene and unique distribution but each of them shares high homologous (33 % – 52 %)[1] and each subtypes receptor always exists simultaneously in the same tissue. Much about the function and distribution of m_3 mAChR has been reposted. For example, it is known that m_3 mAChR is mostly present in smooth muscle and eye, but not in heart[2]. Location of mRNA or changes in mRNA levels may or may not accurately reflect the status of the receptor protein itself[3]. Muscarinic receptors are lacking in full subtype-selective ligands[4].

Antibody can provide direct means to quantitate receptor proteins, and the immunological methodologies afford high sensitivity and selectivity. Antisera of M-receptor subtypes have been used to study the distribution of receptor subtypes by immunoprecipitation, immunohistochemistry, and immunoblotting[5–7]. Monoclonal antibody of anti-peptide m_3-receptor has not been produced.

In this study, we attempted to synthesize a special peptide of m_3 mAChR as an antigen to raise monoclonal antibody against m_3 receptor, and identify its specificity.

MATERIALS AND METHODS

Animals Balb/c mice (7 – 8-wk old, Grade II) and Sprague Dawley rats (250 – 350 g, Certificate № 2698A001) were purchased from Experimental Animal Center, Sun Yat-Sen University of Medical Sciences.

Reagents Keyhole limpet hemocyanin (KLH), Sephadex G-50 and PEG-20000 were purchased from Sigma Chemical Co. Horseradish peroxidase sheep-anti-mouse IgG (HRP-IgG) and FITC sheep-anti-mouse IgG were purchased from Sino-American Biotechnology Co. Mouse Antibody Isotyping Kit was purchased from Life Technologies. LSAB Kit was purchased from Maixing Co. ^{3}H-QNB 1.6095 PBq/mol and ^{3}H-PZ 2.4 PBq/mol were purchased from Amershan Radio Chemical Center.

Preparation and purification of antigen The peptide SSWIHSPSDAGLPPGTVTH of human m_3 receptor was synthesized by multiple peptide system La, Jolla CA according to selected amino acid sequence[8]. The synthetic peptide was purified by reversed-phase high performance liquid chromatography (HPLC), and then

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lyophilized and stored at −20 ℃.

Coupling to KLH[9] KLH 5.8 mg was coupled to 3.3 mg m_3 peptide through 2.6 mg EDAC for 4 h with constantly shaking. The reaction product KLH-m_3 peptide was passed through Sephadex G-50 column equilibrated with PBS 50 mmol/L (pH 7.2) to remove free EDAC. KLH-peptide recovery from peak fractions of the column (monitored by absorbence at 280 nm) was estimated to be 75 %, and then concentrated to 2 g/L by PEG-20000 and stored at −20 ℃.

Preparation and purification of monoclonal antibody (McAb) The McAb was prepared as previously described (Kohler 1975). The KLH-m_3 receptor peptide was injected subcutaneously into Balb/c mouse. The spleen was fused with SP2/0 myeloma cells by following the procedure of Galfrey.

Isotypes of the antibodies were determined using a mouse monoclonal antibody isotyping kit.

Antibody was purified from ascites of mouse vaccinated by hybridoma cells using a combination of two-step precipitation methods and ion-exchange chromatography. Firstly, buffer was added to ascites, followed by 6 % PEG-6000 to precipitate immuno-globulin. Secondly, precipitated immunoglobulin was dissolved in the same buffer and precipitated again. Finally, immunoglobulin was further purified using Sephadex G-50 ion exchange column chromatography, pH of washed fluid was 8.8, Tris-HCl buffer 0.05 mol/L contained NaCl 0.5 mol/L. Purification of the antibody was identified by PAGE polyacryamide gel electrophoresis and SDS-PAGE polyacryamide gel electrophoresis.

Identification of specificity of the monoclonal antibody

Enzyme-linked immunosorbent assay (ELISA) ELISA method A: The microtiter plate was coated with diluted antigen solutions (25 μg m_3 receptor peptide in $Na_2CO_3/NaHCO_3$ 0.05 mol/L, pH 9.5) and blocked by blocking buffer (2 % BSA, wt/vol, 0.4 % Tween-100 vol/vol, in PBS 50 mmol/L, pH 7.4). After being washed, the culture supernatant was added to the plate and incubated at 37 ℃ for 1−1.5 h. After wash, the diluted HRP-IgG was added to each well and incubated at 37 ℃ for 1 h. Then TMB, the cell which has blue colour and which can secret monoclonal antibody was added. ELISA method B: The microtiter plate was coated respectively with protein of rat salivary gland, rat cortex, and m_4 peptide. The rest of the procedure is the same as above.

Radioligand binding assay (RAD) Specific ^{3}H-QNB binding was measured with membrane protein (0.7 mg salivary gland, 0.2 mg heart) at 37 ℃ in TE buffer. Nonspecific binding was defined as ^{3}H-QNB binding in the presence of atropine. Saturation curves were generated by incubating with ^{3}H-QNB 0.3−6 nmol/L. The dissociation constant (K_d) and maximal binding capacity (B_{max}) values were calculated from saturation isotherms. For competition studies, membrane proteins were incubated first with monoclonal antibody at 30 ℃ for 60 min, and then with ^{3}H-QNB 2 nmol/L at 37 ℃ for 30 min.

Histochemistry The tissues of salivary gland and heart were immersed in 4 % paraformaldehyde, and were routinely processed for paraffin embedding. Nonspecific peroxidase activity was inhibited with a 10-min incubation in 0.33 % H_2O_2-methnol and in normal goat serum diluted at 1:20. Sections were incubated with monoclonal antibody at 1:100 dilution at 4 ℃ overnight. The sections were washed and incubated with biotinylated goat anti-mouse IgG for 40 min at room temperature, and then were rinsed with PBS before the third incubation (2 h at RT) in HRP-conjugate streptavidin. After wash, the substrate solution containing DAB, 0.03 % H_2O_2 in Tris-HCl 50 mmol/L, pH 7.6 was added. The reaction was stopped by removing the substrate which was then rinsed with PBS, and cover slipped. Negative control section received identical treatment except for the primary monoclonal antibody part.

RESULTS

Monoclonal antibody With the KLH-m_3 peptide, we succeeded to establish three hybridoma cell lines secreting anti-m_3 peptide antibodies, ie McAbm_{3a}, m_{3b}, m_{3c}. As all three of these showed similar immunohistochemical staining pattern, McAbm_{3b}, that had the highest antibody activity was used in the rest of the study. McAbm_{3b} was IgM, and its titer was 1:1 000 000.

Purification Purified McAbm_{3b} showed one zone in PAGE polycryamide gel electrophoresis. In reduced SDS-PAGE polycryamide gel electrophoresis, purified product showed two chains, ie 71 kDa and 23 kDa which were close to heavy and light chains of IgM (Fig 1). The concentration of the IgM measured by ultraviolet spectrophotometry was 96.3 %.

Specificity

ELISA The results showed that McAbm_{3b} could

specially bind with rat salivary glands and rat cerebral cortex membrane proteins, but not with m_4 peptide. McAbm$_{3b}$ had no cross reaction with m_4 peptide (Fig 2).

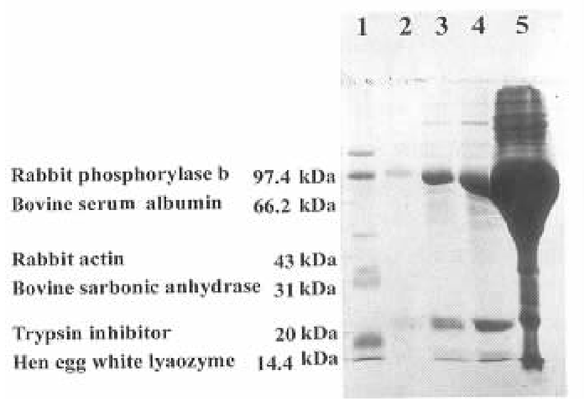

Fig 1. SDS-PAGE analysis of mouse vaccinated with hybridomas prepared at progressive stages of purification. Lane 1: standard molecular weight marker; Lane 2: purified McAbm$_{3b}$; Lane 3: 6 % PEG-solubilized precipitate one; Lane 4: 6 % PEG-solubilized precipitate two; Lane 5: ascites of mice vaccinated with hybridomas cells.

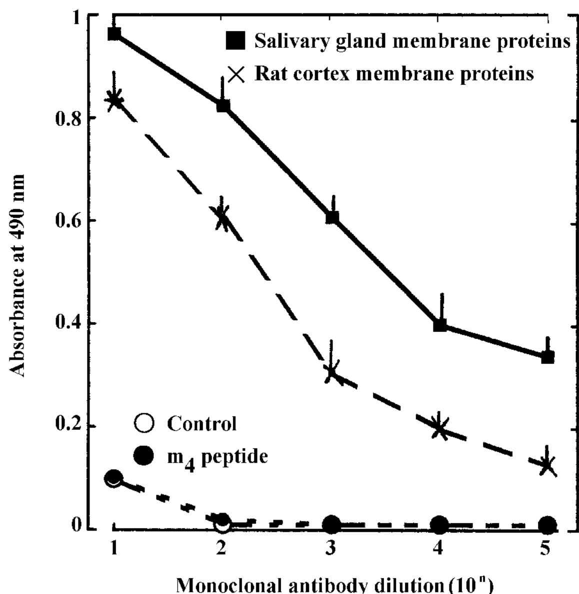

Fig 2. ELISA of m_3 monoclonal antibody with m_4 peptide (20 mg/L), rat cortex membrane proteins (20 mg/L), and salivary gland membrane proteins (20 mg/L) as antigen. Open circle data were from three experiments.

Radioligand binding assay In the salivary gland (m_3 receptor) and heart (m_2 receptor), ^{3}H-QNB bound in a saturated manner having concentrations of 0.8 nmol/L and 0.4 nmol/L, respectively. Scatchard plots indicated a K_d for ^{3}H-QNB of (0.28 ± 0.1) nmol/L and a B_{max} of (402 ± 6) pmol/g protein in the salivary gland, K_d for ^{3}H-QNB of (0.07 ± 0.01) nmol/L and a B_{max} of (504 ± 9) pmol/g protein in the heart.

McAbm$_{3b}$ inhibited the binding of ^{3}H-QNB with the M-receptor in salivary gland protein with inhibition ratio of 43.4 %, but it has no inhibitory effects on heart binding with ^{3}H-QNB with inhibition ratio of 0.075 %. The two ratios exhibited significant difference ($P < 0.01$). The results showed that McAbm$_{3b}$ could not bind with m_2 receptor, ie had no cross reaction with m_2 receptor.

In brain cortex, ^{3}H-PZ (m_1 selective ligand) also bound in a saturated manner with K_d of 4.8 nmol/L and B_{max} of 1107 pmol/g protein. In competitive assay, m_1 antisera could inhibit the ^{3}H-PZ binding with brain cortex protein, but McAbm$_{3b}$ could not (inhibition ratios were 33.3 % and 4.53 %, respectively). The two ratios exhibited notable significant difference ($P < 0.05$). The results showed that McAbm$_{3b}$ could not bind with m_1 receptor, ie had no cross reaction with m_1 receptor.

Histochemistry The cell immunoreactivities of salivary gland tissue and heart tissue were compared. The membrane of salivary gland was densely stained. The membrane of heart was sparely stained in immunohistochemistry. This result showed that McAbm$_{3b}$ had no cross reaction with m_1 and m_2 receptors, α and β receptors, or protein in heart (Fig 3).

DISCUSSION

In the present studies, Balb/c mice were immunized with synthesized subtype-selective peptide segments of m_3 receptor and hybridoma technology was used to develop monoclonal antibody. McAbm$_{3b}$ purified from ascites of mouse vaccinated by hybridoma cells showed high purification (96.3 %) and high titer (1:10 000) with concentration of 0.2 g/L.

ELISA assay showed that McAbm$_{3b}$ could specially bind with rat cerebral cortex membrane proteins and rat salivary glands membrane proteins, but not with m_4 receptor peptide. McAbm$_{3b}$ had no cross-reactivity with m_4 receptor.

RAD showed that maximal inhibition percentage of McAbm$_{3b}$ in rat myocardium and rat salivary glands was 0.075 % and 43.4 %, respectively. This indicated that the m_2 receptor was present almost only in heart cells.

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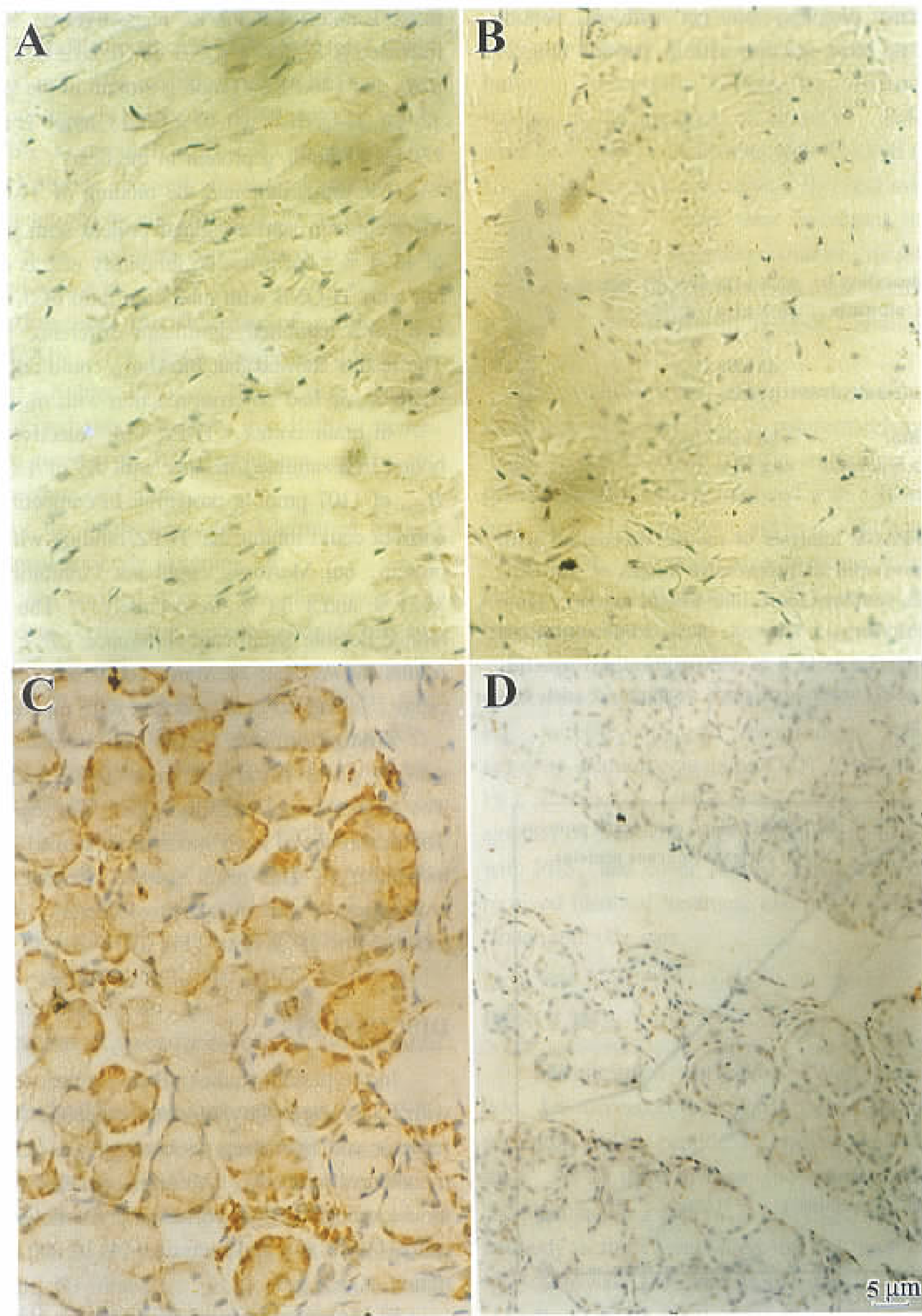

Fig 3. Immunohistochemical localization of m_3 receptor in human heart. No staining was observed in slice with m_3 monoclonal antibody (A), and also no staining in control (B). Immunohistochemical localization of m_3 receptor in human saliary gland. Membranes of ascini was stained (C), No staining was observed in control (D). ×200.

Sharma *et al* found that m_2 receptor but not m_3 receptor mRNA proteins were also present on the surface of heart cells[10]. $McAbm_{3b}$ has no cross-reactivity with m_2 receptor. The maximal inhibition percentage of $McAbm_{3b}$ and m_1-receptor antiserum in rat cortex is 4.53 % and 33.3 %, respectively. It indicates that $McAbm_{3b}$ could not inhibit the ^{3}H-PZ binding to m_1 receptor and had no cross-reactivity with m_1 receptor.

Immunocytochemical experiments with $McAbm_{3b}$ in human myocardium tissue and salivary glands tissue revealed that the cells of salivary glands were intensely stained, whereas there was no staining of the cells of myocardium.

These results indicate that $McAbm_{3b}$ could specifically bind with m_3 receptor, but not m_1, m_2, m_4 receptor, α, β receptors, and other glucoproteins in the human heart. Antipeptide monoclonal antibody of m_3 receptor has high specificity and is a new tool to study the distribution and the function of muscrinic m_3 receptor.

REFERENCES

1 Kerlavage AR, Fraser CM, Venter JC. Muscarinic cholinergic receptor structures: molecular biological support for subtypes. Trends Pharmacol Sci 1987; 426–31.
2 Culp DJ, Graham LA, Latchney LR, Hand AR. Rat sublingual glands as a model to study glandular mucous cell secretion. Am J Physiol 1991; 260 (6 Pt 1): C1233–44.
3 Levery AI, Brann MR. Immunological detection of muscarinic receptors subtype proteins (m_1-m_5) in rabbit peripheral tissues. Mol Pharmacol 1991; 40: 459–62.
4 Hulme EC, Birdrall NJM, Buckley NJ. Muscrinic receptor subtypes. Annu Rev Pharmacol Toxicol 1990; 30: 633–73.
5 Blake AD, Anthony NM, Chen HH, Harrison JB, Nathanson NM, Sattelle DB. Drosophila nervous system muscrinic acetylcocholine receptor: transient functional expression and localization by immunocytochemistry. Mol Pharmacol 1993; 44: 716–24.
6 Yasuda RP, Ciesla W, Flazes LR, Wall SJ, Li M, Satkus SA *et al*. Development of antisera selective for m_4 and m_5 muscarinic cholinergic receptor: distribution of m_4 and m_5 receptor in cat brain. Mol Pharmacol 1993; 43: 149–57.
7 Stephen JW, Robert PY, Min L, Barry BW. Development of an antiserum against m_3 muscacrinic receptor: distribution of m_3 receptor in rat tissues and clonal cell. Mol Pharmacol 1988; 34: 327–33.
8 Bonner TI. The molecular basis of muscarinic receptor diversity. Trends Neurosci 1989; 12: 148–51.
9 Lei S, Rftery MA, Conti-Tronoconi BM. Anti-peptide antibody recognizing native nicotinic AChR. Biochemistry 1993; 32: 90–100.
10 Sharma VK, Colecraft HM, Rubin LE, Sheu SS. Does mammalian heart contain only the m_2 muscarinic receptor subtype? Life Sci 1997; 60: 1023–9.

人 m_3 受体单克隆抗体的研制[1]

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关键词　毒蕈碱受体；单克隆抗体；酶联免疫吸附测定；放射配位体测定；免疫组织化学

目的：制备抗人 m_3 受体肽链的单克隆抗体. 方法：人工合成人 m_3 受体亚型特异的细胞外氨基端肽链段，与血蓝蛋白偶联，免疫 Balb/c 小鼠，制备 m_3 受体的单克隆抗体. 用 PEG-6000 二步沉淀法与 Sephadex G-50 柱层析分离纯化抗体. 用 ELISA、免疫组织化学和放射配基法鉴定抗体的特异性. 结果：抗体与 m_3 肽链、大鼠唾液腺及大脑皮质膜蛋白发生特异性的结合，不与 m_4 肽链发生结合. 抗体能抑制^{3}H-QNB 与大鼠唾液腺膜蛋白的结合，不抑制^{3}H-QNB 与大鼠心肌的结合，也不抑制^{3}H-PZ 与大鼠大脑皮质膜蛋白结合. 免疫组织化学结果显示唾液腺呈棕色阳性反应，而心肌呈阴性反应. 结论：本研究制备的人 m_3 受体单克隆抗体具有高的特异性，可专一性识别 m_3 受体.

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