A Monoclonal Anti-peptide Antibody against β2-adrenergic Receptor Which Specifically Binds [^{3}H]dihydroalprenolol

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Abstract—The analysis of membrane receptors for hormones and neurotransmitters has progressed considerably by pharmacological and biochemical means and more recently through the use of specific antibodies. To generate and characterize a moloclonal antibody against β-adrenergic receptor, a synthetic β2-adrenergic receptor peptide (Phe-Gly-Asn-Phe-Trp-Cys-Phe-Trp-Thr-Ser-Ile-Asp-Val-Leu) which may comprise part of β-adrenergic receptor ligand binding pocket was coupled to Keyhole Limpet Hemocyanin (KLH) and used as an immunogen. Male BALB/C mice were immunized with this antigen and the immunized spleen was fused with myeloma SP2/0-Ag14 cells to produce monoclonal antibodies. Two clones were obtained but one of monoclonal antibodies, mAb5G09, was used throughout in this study because the other clone, mAb5A11 showed weak immunoreactivity against KLH as well. The mouse monoclonal antibody mAb5G09 produced in this study showed immunoreactivity to peptide-KLH conjugates and also to human A431 cells and guinea pig lung β2-adrenergic receptor as revealed by ELISA and western blot. In the course of determination of the effects of mAb5G09 on β-receptor ligand binding, it was observed that mAb5G09 specifically bound β-adrenergic radioligand [^{3}H]dihydroalprenolol (DHA) with a dissociation constant (Kd) of 60 nM. The [^{3}H]DHA binding activity of mAb5G09 had characteristics of immunoglobulins and the binding activity was not observed in the control anti-KLH monoclonal antibody. The monoclonal antibody, mAb5G09 produced in this study may provide useful models for the study of the structure of receptor binding sites.

Keywords □ β2-adrenergic receptor, a synthetic peptide, [^{3}H]dihydroalprenolol, mAb5G09, ligand binding

The adenylate cyclase-coupled β-adrenergic receptors have been thought as a model system for the investigation of receptor-ligand interaction and receptor mediated signal transduction mechanism. Over the past decades, the analysis of membrane receptors for hormones and neurotransmitters such as β-adrenergic receptor, has progressed considerably by pharmacological (Dohlman *et al.*, 1991) and biochemical means (Ostrowski *et al.*, 1992) and more recently through the use of specific antibodies (Bahouth *et al.*, 1991).

In order to characterize these receptors it was of interest to raise specific antibodies to the receptors. Initially, antibodies against membrane receptors including β-adrenergic receptor have been generated against partially purified receptors (Weiss *et al.*, 1987; Moxham *et al.*, 1986) or found in the circulation of patients with specific diseases (Venter *et al.*, 1980). Antibodies against β-adrenergic receptor have been used for the wide range of studies including receptor structure (Moxham *et al.*, 1986, 1988), topography (Theveniau *et al.*, 1989; Wang *et al.*, 1989a) and distribution in the brain (Wanaka *et al.*, 1989) and cell (Wang *et al.*, 1989b). Generally, receptor molecules exist only in minute quantity in nature (about fmol range per mg protein), so the production of antibodies directed against synthetic peptide seems to be one of the reasonable approaches to obtain useful biochemical tools for the investigation of β-adrenergic receptor (Cheung *et al.*, 1991; Palm *et al.*, 1990).

The hydrophobic regions of the β-adrenergic receptor which might form transmembrane helices are thought to be important in receptor ligand binding (Dixon

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et al., 1987). The prime candidates of amino acids which were important for ligand binding were considered as several hydrophilic groups in transmembrane region. Especially, Asp-113 residue in the third transmembrane helix of β-adrenergic receptor was thought to act as the counterion for the cationic amino group of the adrenergic ligands (Strader *et al.*, 1988).

In the course of production and characterization of a monoclonal antibody against a β-adrenergic receptor synthetic peptide in which Asp-113 residue was contained, it was observed that this antibody specifically binds β-adrenergic ligand [^{3}H]dihydroalprenolol as well as β-adrenergic receptor itself. The properties of this unique monoclonal antibody and its implications for the study of hormone and neurotransmitter receptor structure and function will be discribed in this report.

Materials and Methods

Materials

(−)-[^{3}H]Dihydroalprenolol ([^{3}H]DHA, 76 Ci/mmol) was purchased from Amersham(Aylesbury, UK). Whatman GF/B filters were purchased from Fisher (Pittsburgh, PA). Electrophoresis reagents were obtained from Bethesda Research Laboratories (Gaithersburg, MD). Freund's Adjuvants and other immunochemical reagents were purchased from Pierce (Rockford, IL). Reagents for the hybridoma cell culture and myeloma-spleen cell fusion including polyethylenglycol, HT (hypoxanthin and thymidine) and HAT (hypoxanthine, aminopterine and thymidine) media supplements were obtained from Sigma (St. Louis, MO). Pre-stained protein molecular weight marker and iron supplemented calf serum were obtained from Gibco (Gaithersburg, MD). All other reagents were obtained from Sigma Chemicals (St. Louis, MO) and were of the highest commercially available grade.

Immunization

The peptide used to construct the immunogen was the residues (102-115) of the human β2-adrenergic receptor (Dixon *et al.*, 1986). The peptide sequence was Phe-Gly-Asn-Phe-Trp-Cys-Phe-Trp-Thr-Ser-Ile-Asp-Val-Leu (residue 102-115, 14mer). The peptide was synthesized by the Laboratory of Protein Chemistry, KIST Genetic Engineering Institute and was purified by HPLC. The peptide was conjugated to keyhole limpet hemocyanin (KLH) by glutaraldehyde method and the peptide-KLH conjugate was dialyzed overnight against excess PBS. Male BALB/C mice (8 weeks of age) were immunized with an emulsion of 0.2 m*l* of the peptide-KLH (1 mg protein /m*l*) in an equal volume of Complete Freund's Adjuvant (CFA) via intraperitoneal route. Booster immunization were performed for two times at 3 weeks interval with Incomplete Freund's Adjuvant (IFA). Three days before the spleen and myeloma cell fusion, the animal was injected with 0.4 ml immunogen (KLH-peptide) alone.

Production of Monoclonal Antibody

Immunized mice were sacrificed by cervical dislocation and the spleen cells were aseptically removed in the laminar flow. The spleen cells were mixed with equal volume of SP2/0-Ag14 myeloma cell (about 4×10^7 cells) and harvested by centrifugation at 4℃. The mixture was washed two times with 40 m*l* of incomplete Dulbecco's Modified Eagle's medium (DMEM) and the two cell population were fused with dropwise addition of 1 m*l* of 50% polyethylenglycol 1,500 over 1 minute at 37℃. The reaction went on additional 90 seconds and the reaction was terminated by addition of 20 m*l* of incomplete DMEM. The fused cells were resuspended in complete DMEM/ 20% iron supplemented calf serum culture media containing hypoxanthine, aminopterine, thymidine (HAT) selection reagent and 100 μ*l* aliqouts of the cell suspension were distributed into five 96 well culture plate which contained 100 μ*l* of peritoneal washout feeder layer. After selection the fused cells were maintained in hypoxanthine, thymidine (HT) propagation media until 50% confluency was reached. The hybridomas were screened by dot blot and ELISA. Positive hybridomas were expanded into 24 well culture plates and T-25 culture flasks stepwise. The hybridomas were cloned by two consecutive rounds of limitting dilution methods.

Dot Blot Assay

Antigens (Peptide-KLH, KLH, Bovine Serum Albumin (BSA), Peptide-BSA and Peptide, 1 μg each) were blotted on nitrocellulose membrane. Strips were blocked with 5% non-fat dried milk, 0.2% Tween 20 in PBS. Hybridoma culture supernatants diluted 1 : 5 in blocking solution were added to the strips and incubated for 1 hour. After rinsing, strips were incubated with peroxidase-labeled goat anti-mouse IgG antibody (Pierce; Rockford, IL, crossreactive also to mouse IgM) diluted 1 : 5,000 in blocking solution. After rinsing, strips were then visualized with substrate solution (0.6 mg/m*l* 3,3'-Diaminobenzidine in 50 mM Tris, pH 7.6, 0.05% (v/v) H_2O_2).

Enzyme-linked Immunosorbent Assay (ELISA)

Peptide-KLH congugates (50 ng) diluted in PBS was added to wells of microtiter assay plate (Falcon) and allowed to adsorb for 1 hour at room temperature. In some cases, human epidermoid carcinoma cell line

A431 (obtained from ATCC) was cultured on 96 well culture plate and used as antigen. After Blocking, the wells were then incubated 1 hour at room temperature with 50 μl of monoclonal antibody culture supernatants or ascites fluids of the monoclonal antibody which were serially diluted in blocking solution. The wells were then probed with 50 μl of peroxidase-labeled goat anti-mouse IgG antibody diluted 1 : 5,000 in blocking solution. Each well was developed with 50 μl of soluble substrate solution (0.01 mg Tetramethylbenzidine/ml 0.1 M sodium acetate, pH 6.0, 0.01% H_2O_2) and the absorbance was read at 450 nm.

Immunoblot Assay

After SDS-polyacrylamide gel electrophoresis of human A431 membrane preparation (50 μg protein/lane) which naturally expresses β2-adrenergic receptor and 1 μg of partially purified guinea pig lung β2-adrenergic receptor (specific activity of 1.2 nmol/mg protein, prepared with minor modification according to the procedures described by Benovic *et al.* (1985)), electrophoretic transfer of the proteins was performed in methanol/glycine/Tris buffer as described (Towbin *et al.*, 1979). After blocking, nitrocellulose strips were incubated for 2 hour at room temperature with ascites fluids diluted 1 : 200 in blocking solution. After washing, they were incubated with peroxidase-labeled goat anti-mouse IgG diluted 1 : 5,000 in blocking solution for 1 hour at room temperature. After washing, strips were then visualized with diaminobenzidine substrate solution.

[^{3}H]Dihydroalprenolol Ligand Binding Assay

Monoclonal antibody culture supernatants or ascitic fluids which were dialyzed against phosphate buffered saline(PBS) were incubated with varying concentration of [^{3}H]DHA for 60 minutes at 23℃. For the examination of the effects of monoclonal antibody on receptor ligand binding, mAb5G09 preparation were added to guinea pig lung β-adrenergic receptor and [^{3}H]DHA binding mixture. Ligand bound complex was separated from free ligand by vacuum filtration through 0.5% polyethylenimine (PEI) pretreated Whatman GF/B filters and the radioactivity was determined by liquid scintillation counting. Nonspecific binding was defined as that binding not inhibited by 10 μM *dl*-propranolol. In saturation binding assay, the data were analysed with Ligand computer program.

Results

Preparation and Characterization of Monoclonal Antibody

In the course of preparation of anti-peptide monoclonal antibody, finally two clones of monoclonal antibodies were obtained. These monoclonal antibodies named as mAb5G09 and mAb5A11 respectively. For initial characterization of these monoclonal antibodies, dot

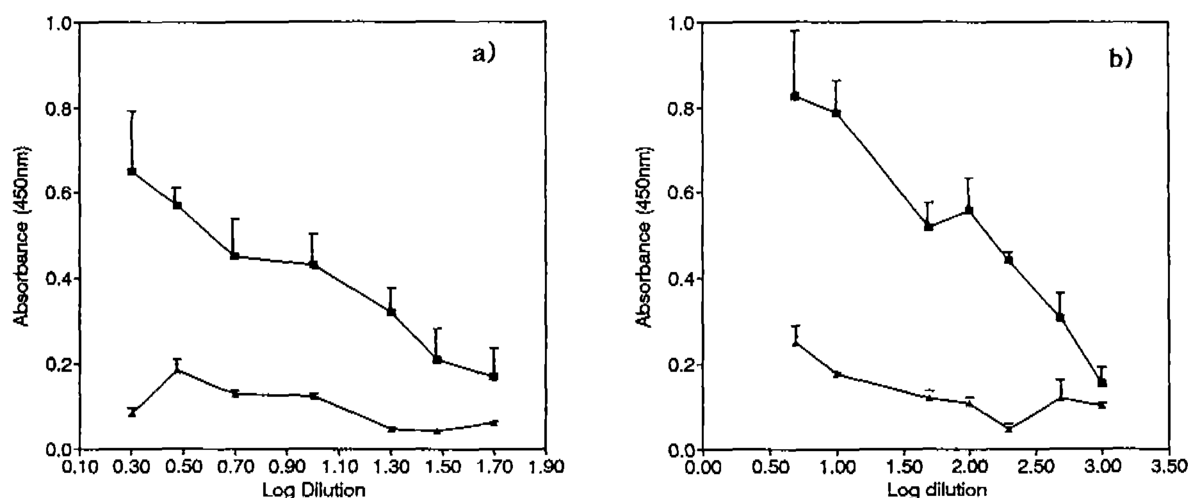

Fig. 1. ELISA of human A431 cells with mAb5G09. Human A431 cells which naturally expresse β2-adrenergic receptor were cultured in 96 well culture plate (4×10^4 cells/well). After fixing with 1 : 1 mixture of aceton and methanol at room temperature for 3 minutes the plates were extensively washed with phosphate buffered saline (PBS). Each well was incubated with a) serially diluted mAb5G09 culture supernatants or b) ascitic fluids of mAb5G09 for 1 hour at room temperature. The wells were probed with horse radish peroxidase (HRP) labelled goat anti-mouse IgG for 1 hour at room temperature (diluted 1 : 5,000 in PBS-0.2% tween). 3,3',5,5'-tetramethylbenzidine was used for soluble substrates and the absorbance was read at 450 nm. The data shown here are the means± S.D. from four such experiments each done in triplicate. ■; mAb5G09, ▲; Control anti-KLH mAb.

blot and ELISA were carried out using KLH and KLH-peptide as antigens. Both monoclonal antibodies show immunoreactivity against KLH-peptide but in the case of mAb5A11, there is weak immunoreactivity against carrier protein KLH itself as well. So, we further characterized only mAb5G09 afterward. The isotype of mAb5G09 was IgM (determined with Sigma isotyping kit). As shown in Fig. 1, mAb5G09 showed immunoreactivity to human epidermoid carcinoma cell line A 431 which naturally expresses β2-adrenergic receptor. To identify whether this immunoreactivity was actually to β2-adrenergic receptor, western blot was conducted. As shown in Fig. 2, mAb5G09 revealed a protein band of Mw 64,000. Also, mAb5G09 revealed a protein band of Mw 64,000 in western blot of partially purified β-adrenergic receptor (Fig. 2) which was obtained from guinea pig lung, indicating cross reactivity toward guinea pig β2-adrenergic receptor as well as human β2-adrenergic receptor.

[^{3}H]Dihydroalprenolol Binding Activity of Monoclonal antibody

To investigate the effect of mAb5G09 on the β-adrenergic receptor ligand interaction, varying amounts of culture supernatants or ascitic fluids of mAb5G09 were added to the β-adrenergic receptor and [^{3}H]DHA binding mixture and the specific radioligand binding was determined. In contrast to initial expectation, both culture supernatants and ascitic fluids of mAb5G09 increased the specific binding of [^{3}H]DHA to guinea pig lung soluble β2-adrenergic receptors about 40% in a concentration-dependent manner (Fig. 3) while control supernatants or ascitic fluids were without effect. To determine whether this increase in [^{3}H]DHA binding was due to the direct binding of [^{3}H]DHA to mAb5G 09, saturation binding assay was performed. mAb5G09 specifically bound [^{3}H]DHA with a Bmax of 16.2 pmol/m*l* culture supernatant and dissociation constant (Kd) was about 60 nM. The binding activity has characteristics of immunoglobulin as evidenced by complete recovery of binding activity in the 50% ammonium sulphate precipitatable fraction (Fig. 4) and resistance to heat inactivation (Fig. 5).

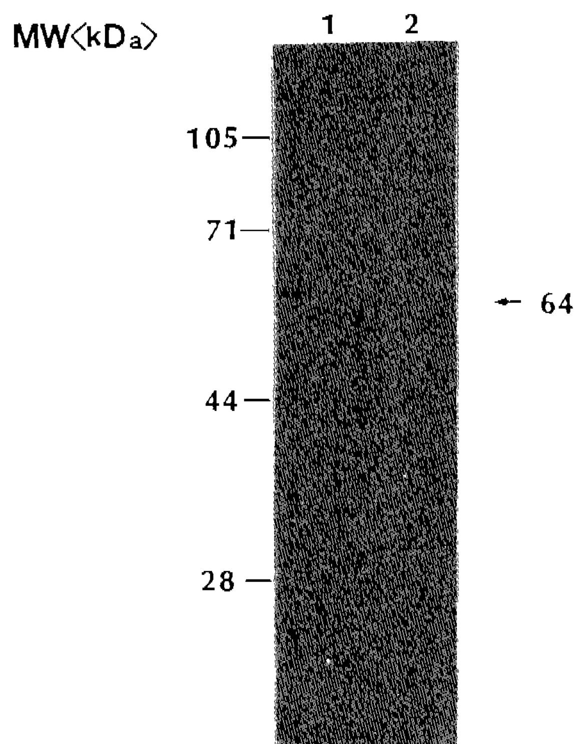

Fig. 2. Western blot of β2-adrenergic receptor with mAb5G 09. A431 cell membrane preparation (lane 1, 50 μg) and partially purified guinea pig lung β2-adrenergic receptor (lane 2, 1 μg) were electrophoresed in 10% SDS-PAGE and the protein bands were electrically transferred to nitrocellulose membrane. The strip was incubated with 1 : 200 diluted ascitic fluids of MAb5G09 for 2 hours at room temperature and probed with horse radish peroxidase (HRP) labelled goat anti-mouse IgG for 1 hour at room temperature (diluted 1 : 5, 000 in PBS-0.2% tween). The bands were visualized with diaminobenzidine. The 64 kDa band of β2-adrenergic receptor was indicated by an arrow.

Discussion

In the present study the anti-peptide monoclonal antibodies showed immunoreactivity against KLH-peptide and also against human and guinea pig lung β-adrenergic receptor. In the western blot, mAb5G09 showed a single band of Mw 64 kDa which was identical to the previously reported molecular weight of β2-adrenergic receptor (Wang *et al.*, 1989a,b; Kaveri *et al.*, 1987). To determine the possible effects of mAb5G09 on receptor ligand ([^{3}H]DHA) interaction, mAb5G09 was included in the β2-adrenergic receptor [^{3}H]DHA binding mixture and this causes dose dependent increase of [^{3}H]DHA binding. This increase in binding activity was due to the direct binding of [^{3}H]DHA to mAb5G09. In saturation binding experiment, mAb5G 09 specifically bound [^{3}H]DHA with a Kd value of 60 nM. One can speculate that the binding activity may arise from myeloma cell membrane which might contaminate mAb5G09 culture supernatant or from calf serum present in culture media. Several lines of evidence demonstrate that this was not the case. First, the binding maximum (16.2 pmol/m*l* culture supernatant) was at least 1,000 fold higher than that of myeloma SP2/0-Ag14 cells in culture (about 15 fmol/m*l*

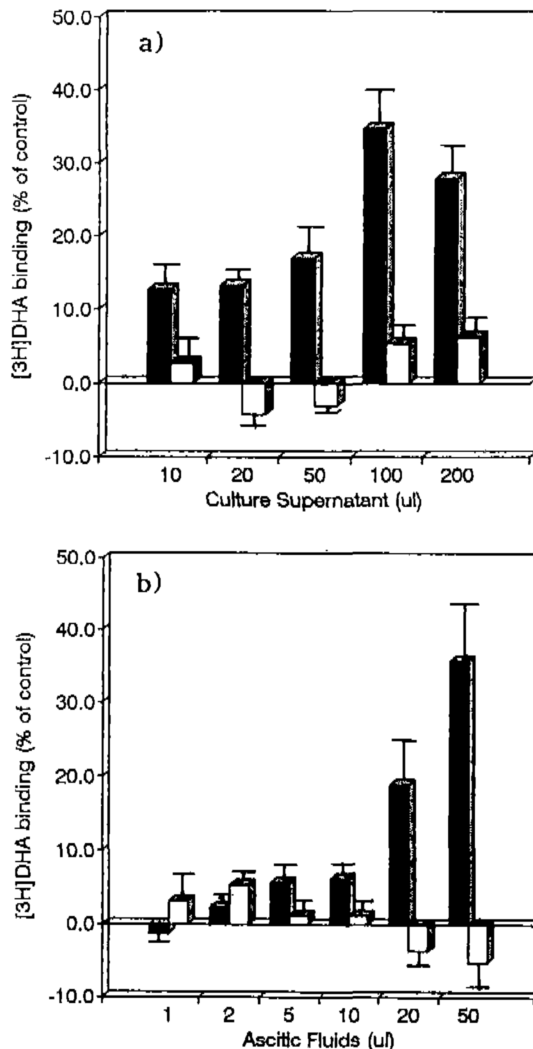

Fig. 3. Effects of mAb5G09 on β-adrenergic receptor ligand binding. Guinea pig lung β2-adrenergic receptor was solubilized with 1.5% digitonin and an aliquot of the solubilized receptor was incubated in a total assay volume of 0.5 ml with varying amounts of mAb5G09 which was extensively dialyzed against PBS (black box). After 10 minutes, the ligand binding reaction was initiated with the addition of 2 nM [^{3}H]dihydroalprenolol. The binding mixture was incubated for 30 minutes at 23℃ and the reaction was terminated by vacuum filtration. The radioactivity on GF/B filter was determined by liquid scintillation counting. Nonspecific binding was determined with 10 μM dl-propranolol. Radioligand binding when there is no antibody addition was regarded as initial 100% binding (average 2,300 cpm± 78). A monoclonal anti-KLH antibody was used as control antibody (empty box). The data shown here are the means± S.D. from three such experiments each done in triplicates. a) Culture supernatants b) ascitic fluids.

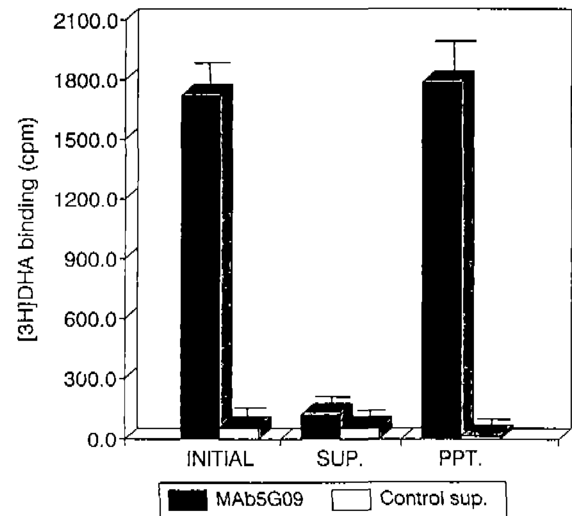

Fig. 4. Precipitation of [^{3}H]DHA binding activity of mAb5G 09 by 50% ammonium sulfate. mAb5G09 culture supernatant was precipitated with dropwise addition of saturated ammonium sulfate to a concentration of 50%. After 4 hours at 4℃, the precipitates were recovered by centrifugation (10,000 xg, 10 minutes) and reconstituted in an original volume of PBS. The precipitates and supernatant were extensively dialyzed against PBS and the [^{3}H]DHA binding activity was measured at a radioligand concentration of 10 nM. Almost all the binding activity was recovered from precipitates. The data shown here are the means± S.D. from three such experiments each done in triplicates. A monoclonal anti-KLH antibody was used as control antibody. Initial; untreated culture supernatant. SUP; supernatant fraction of ammonium sulfate tratment PPT; precipitates of ammonium sulfate treatment.

at confluency). Second, control supernatants or ascitic fluids obtained from myeloma cell or monoclonal anti-KLH antibody producing cells did not show any increase in β2-adrenergic receptor [^{3}H]DHA binding activity (Fig. 3) or direct binding with [^{3}H]DHA. Third, high speed centrifugation (100,000 Xg, 1 hour) did not alter the [^{3}H]DHA binding activity of mAb5G09. Also, in the binding study we usually used mAb5G09 preparation which was extensively dialysed or desalted against PBS eliminating possibility of contamination of low molecular weight stimulant of [^{3}H]DHA binding. The [^{3}H]DHA binding activity of mAb5G09 culture supernatants had some characteristics of immunoglobulins. First, the binding activity was completely recovered from 50% ammonium sulphate precipitatable fraction (Fig. 4) while β-adrenergic receptor in animal tissue (e.g. in guinea pig lung) completely lose binding activity after ammonium sulfate precipitation. Second, heat inactivation at 56℃ did not alter the [^{3}H]DHA binding activity of mAb5G09 (Fig. 5). In control experiments, β-adrenergic receptor from human A431 cells or guinea pig lung lose over 80% of their [^{3}H]DHA binding acitivity after 30 minutes heat inactivation at 56℃.

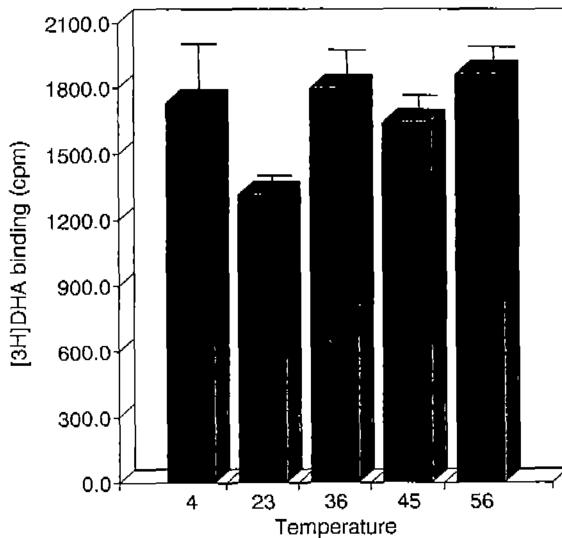

Fig. 5. Heat stability of [^{3}H]DHA binding activity of mAb5G 09. mAb5G09 culture supernatant was incubated at indicated temperature for 30 minutes and the [^{3}H]DHA binding activity was measured at a radioligand concentration of 10 nM. 10 μM dl-propranolol was used to define nonspecific binding. The binding activity was not inhibited by 56℃ heat tratment. The data shown here are the means± S.D. of three separate experiments each done in triplicates.

Third, the [^{3}H]DHA binding activity comigrate with immunoglobulin on Sephadex G-200 column chromatography.

Some investigators reported production of antibodies which specifically bound β-adrenergic ligand including [^{3}H]DHA. In these cases the immunogens were macromolecular conjugates of adrenergic ligand but not β-adrenergic receptor or receptor derived peptide (Rockson *et al.*, 1980; Pitha *et al.*, 1980) or antibodies against β-adrenergic receptor were used as immunogen for the production of 'anti-idiotypic' antibody (Strosberg, 1989). However, the unique property of mAb5G09 produced in this study could not be explained as 'anti-idiotypic' antibody. If it was the case, the antibody produced in this study would not show immunoreactivity against β-adrenergic receptor.

It was reported that polyclonal antibodies directed against purified β-adrenergic receptor from frog erythrocyte specifically bound β-adrenergic ligand as well as frog erythrocyte β-adrenergic receptor itself (Caron *et al.*, 1979). In this report, the authors suggested that the likely immunogen would be the noncovalently associated β-adrenergic receptor ligand complex. But, in the present study the antigen was synthetic peptide which was covalently attached to irrelevant carrier protein KLH and there is no reason to assume that endogeneous β-adrenergic ligand noncovalently attach to peptide-KLH conjugates.

At present, it is most reasonable to assume that mAb 5G09 has an internal image of antigen which might be at least part of β-adrenergic receptor ligand binding pocket and also has a binding site for the antigen; amino acid 102-115 residue of human β-adrenergic receptor.

The binding affinity of [^{3}H]DHA to mAb5G09 (Kd = 60 nM) was somewhat lower than that to the natural β-adrenergic receptor (Kd = 1~2 nM). However, availability of mAb5G09 in soluble form to a practically limitless amounts render the possibility that after purification mAb5G09 might be used as a model for the study of β-adrenergic receptor ligand interaction. In some of human diseases including asthma circulating auto-antibodies against β-adrenergic receptor were detected (Venter *et al.*, 1980). It will be also of interest to identify if there exists catecholamine binding auto-antibodies like mAb5G09 in the serum of asthmatic patients.

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