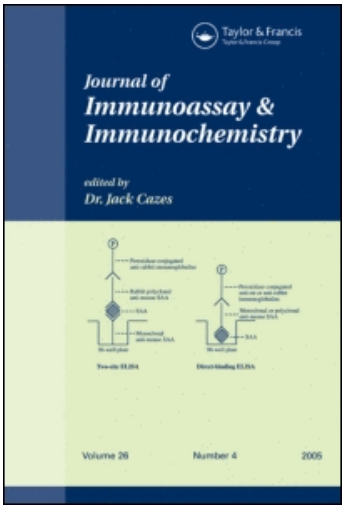

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PRODUCTION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES REACTIVE WITH MELATONIN

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ABSTRACT

Monoclonal antibodies(moAbs) reactive with melatonin(MT) were produced using MT, coupled to bovine serum albumin(BSA) with the Mannich reaction, as immunogen and conventional hybridoma techniques. Hybridoma clones secreting the moAbs were selected by an enzyme-linked immunosorbent assay system using MT-carboxymethylchitin and BSA as screening antigens. The moAbs from 6 clones were characterized by a cross-reactivity test using radioimmunoassay with ^{125}I-labelled MT. The moAbs recognized MT but hardly recognized other analogues except for N-acetylserotonin with a crossreactivity of 0.81%. An inhibition curve for MT was obtained in the range of 50 pg to 100 ng and 1.4 ng of MT inhibited the value of the assay by half. There is interference from some unknown source in human serum.

(KEY WORDS: Melatonin, Monoclonal antibodies, Radioimmunoassay, Immunoassay.)

INTRODUCTION

Melatonin(MT) is a hormone secreted from the pineal gland into the circulation system. The level of MT in the pineal gland and in the blood is circadian-

rhythmic: high during the night-time but very low during the day (1). Therefore, the determination of the MT level has been proposed for estimating the time of death in the field of forensic practice.

Since the MT level in the pineal gland and blood is very low (2,3), radioimmunoassays(RIAs) using polyclonal antibodies have been used exclusively by investigators (4-7). Arendt (8,9) reviewed assays for MT and emphasized the need to improve the specificity of RIA.

Since monoclonal antibodies (moAbs) may improve the specificity achieved with polyclonal antibodies, we describe the production and characterization of moAbs against MT.

MATERIALS AND METHODS

Chemicals

Melatonin, DL-α-methyltryptamine, Nω-methyltryptamine and 5-hydroxy-Nω-methyltryptamine oxalate were purchased from Aldrich Chemical Co., Inc., U.S.A. 5-Methoxytryptamine was obtained from Fluka A G Chemische Fabrik, Buchs, Switzerland and 3-indoleacetic acid, indole, L-histidine, tryptophan, choline chloride, L-aspartic acid, L-glutamic acid and glycine from Wako Pure Chemical Ind., Japan. All other compounds listed

in TABLEs 1 and 2 were purchased from Sigma Chemical Co., U.S.A. [^{3}H]Melatonin(specific activity, 85 Ci/mmol) and 2-[^{125}I]iodomelatonin(specific activity, 2000 Ci/mmol) were supplied by Amersham International plc. CM-Chitin 10C was purchased by Funakoshi Pharmaceutical Co., Japan. Anti-mouse IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgM and IgA antisera were obtained from Serotec, Co., Ltd., U. K. All other reagents were supplied by Nakarai Chemical Co., Ltd., Japan.

Preparation of Antigen Conjugates

(i) An immunogen was prepared by binding MT with [^{3}H] MT to bovine serum albumin(BSA) with the Mannich reaction as prepared by Grota and Brown(10). The degree of conjugation was calculated to be about 10 mol of the hapten per mol BSA(mol. wt. 68,000) on the basis of the radioactivity measured in a liquid scintillation counter (Aloka LSC 903).

(ii) MT-carboxymethylchitin (MT-CM-chitin) was synthesized for moAb screening as follows. Twenty-three mg of 4-amino-D,L-phenylalanine and 2.4 mg of sodium bromide were dissolved in 1 ml of water and acidified with 0.024 ml of conc. hydrochloric acid solution. Then 0.2 ml of water containing 10.8 mg of

sodium nitrite was added dropwise to the mixture and the solution was mixed for 10 min in an ice bath. The excess sodium nitrite was then neutralized with 1% ammonium sulfamate aqueous solution. Thirty mg of MT containing [^{3}H]MT (250,000 dpm) were dissolved in 4 ml of 0.1 M borate buffer (pH 9.0) with 1 ml of dimethylformamide. The diazotized 4-amino-D,L-phenylalanine solution was added to the MT solution. The mixture was stirred for 6 h on the ice bath maintaining pH between 8.0 and 8.5. Thirty mg of CM-Chitin 10C in 1 ml of water and 38 mg of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride in 0.5 ml of water were added to the diazo-coupled MT derivative solution and stirred for 24 h at room temperature maintaining pH between 4.0 and 5.0. A further 19 mg of the carbodiimide were added to the reaction mixture and stirred for 24 h followed by dialysis against running water for 48 h. The degree of conjugation with CM-chitin was calculated to be about 21 mol of the hapten per CM-chitin (mol. wt. 85,000) on the basis of the radioactivity.

Production of MoAbs

A female six-week-old BALB/C mouse was first immunized i.p. with 0.25 mg of MT-BSA in 0.25 ml of

emulsion of saline and Freund's complete adjuvant(1:1 by vol). Thereafter i.p. injections of 0.25 mg MT-BSA in saline were given monthly over 4 months. Three days after the final injection, spleen cells were fused with P3U1 myeloma cells(5:1 by number) using polyethylene glycol 1500 and cultured in the Iscove's modified Dulbecco's medium containing hypoxanthine, aminopterin and thymidine (11). Hybridoma clones secreting moAbs were selected by an enzyme-linked immunosorbent assay system using MT-CM-chitin and BSA as screening antigens (12) except that the MT-CM-chitin was coated on wells by drying 500 ng in 0.05 ml per well at 37 °C for 6 h. Hybridoma cells contained in wells in which culture supernatants were positive to MT-CM-chitin but negative to BSA, were subcloned twice by a limiting dilution method to ensure their monoclonal origins. Isotype analyses were performed by the double-diffusion technique of Ouchterlony(13). MoAb-secreting hybridomas (1×10^8) were injected i. p. into an adult female BALB/C mouse which had been injected i. p. with 0.5 ml of Pristane (Aldrich Chemical Company, Inc., U. S. A.) 3 weeks previously and the ascites produced was used as a source of antibody.

RIA

MoAbs and reagents were diluted in 0.05 M phosphate buffered saline (pH 7.4) containing 2.5%

ethanol and 0.06% bovine gamma globulin. For the preparation of an inhibition curve and for a cross-reactivity test, each assay tube (0.5 ml) contained the following : 0.2 ml of [^{125}I]MT(5000 dpm), 0.2 ml of MT, its analogues or selected compounds and 0.1 ml of the diluted ascites containing moAbs. This assay mixture was incubated for 2 h at room temperature. Antibody-bound [^{125}I]MT was separated from the free by the salting-out method(14), and its radioactivity was measured in an Aloka auto well gamma system(ARC-500).

Serum Samples

Individual sera were collected during the daytime from 20 healthy adult women, pooled and inactivated by incubation at 56 °C for 30 min to destroy complements. Pooled serum (0.1 ml) was added to the RIA system instead of unlabelled MT or related compounds.

RESULTS AND DISCUSSION

Six clones of hybridoma were selected, all secreting moAbs specific for MT-CM-chitin but not BSA. The diluted ascites(1:5000) was shown to bind about 50% of 5000 dpm of [^{125}I]MT by the RIA procedure. The

specificity of each antibody was characterized by the cross-reaction test, in which the moles of each MT analogue or selected compound which gave 50% displacement of the binding of moAb to [^{125}I]MT was compared to that of MT, where the crossreactivity of MT was chosen as 100%. The six moAbs showed almost the same features and representative results are shown in TABLEs 1 and 2. The immunoglobulin isotype of each moAb was tested by the Ouchterlony method and all were identified as IgG_1 but no experiments were done to establish whether the moAbs were identical and recognized the same epitope. Ascitic fluid derived from the clone secreting the highest level of moAb in culture medium was used to examine assay sensitivity.

The inhibition curve for MT was obtained in the range of 50 pg to 100 ng, with 1.4 ng of MT giving 50% inhibition (FIGURE 1). Despite using [^{125}I]MT, our RIA was no more sensitive than those performed by previous investigators using polyclonal antibodies and [^{3}H]MT, e. g., 2 or 25 pg per tube (4) and 40 pg per tube(5) indicating that the antigen binding affinity of our moAb is lower than that of the polyclonal antibodies used (15).

The anti MT moAb obtained was highly specific to MT as shown in TABLEs 1 and 2. The only one slightly crossreacting analogue was N-acetylserotonin, which is

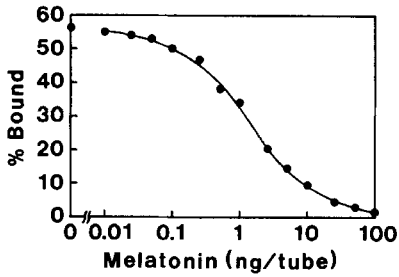

FIGURE 1. Displacement of [^{125} I]MT bound to moAb by unlabelled MT in RIA.

TABLE 1

Crossreactivities of Anti-MT MoAb against MT and its Analogues in RIA Using [^{125} I]MT.

Compounds	% Crossreactivity
Melatonin	100
N-Acetylserotonin	0.81
6-Hydroxymelatonin	0.009
5-Methoxytryptophol	<0.001
5-Hydroxyindole-3-acetic acid	<0.1
5-Methoxytryptamine	<0.1
5-Methyl-3-indoleacetic acid	<0.001
7-Methyltryptamine	0.002
DL-α-Methyltryptamine	<0.001
Nω-Methyltryptamine	<0.001
5-methyltryptamine hydrochloride	<0.001
N-Acetyl-L-tryptophan	<0.001
5-Hydroxytryptophan	<0.1
5-Methoxytryptophan	<0.1
N-Acetyl-L-tryptophanamide	<0.001
5-Hydroxy-Nω-methyltryptamine oxalate	<0.001
5,6-Dihydroxytryptamine creatinine sulfate	<0.001
2-Methyl indole	<0.001
3-Methyl indole	<0.001
5-Methyl indole	<0.001
5-Methoxyindole	0.085
3-Indoleacetic acid	<0.1
Indole	<0.1
Harmane(free base)	0.019
Harmaline hydrochloride	<0.001
Harmine hydrochloride	0.092
6-Methoxyharmalan	0.092

TABLE 2

Crossreactivities of Anti-MT MoAb in RIA Using [^{125} I] MT against Selected Amino Acids, Putative Neurotransmitters and Their Derivatives in Biological Tissues

Compounds	% Crossreactivity
L-Tyrosine	<0.001
Tyramine(free base)	<0.001
3-Hydroxytyramine hydrochloride	<0.001
(-)-Epinephrine	<0.001
DL-Metanephrine hydrochloride	<0.001
γ-Amino-n-butyric acid	<0.001
γ-Hydroxybutyric acid	<0.001
L-Histidine(free base)	<0.001
Histamine dihydrochloride	<0.001
Tryptophan	<0.001
Tryptamine hydrochloride	<0.001
5-Hydroxytryptamine hydrochloride	<0.001
Choline chloride	<0.001
Acetylcholine chloride	0.001
L-Aspartic acid	<0.001
L-Glutamic acid	<0.001
Glycine	<0.001

the precursor of MT in pineal glands of animals. Rabbit polyclonal antiserum against the same immunogen (5) not only showed comparable crossreactivity with N-acetylserotonin(1.3%) to our moAb(0.81%), but also crossreacted with 6-hydroxymelatonin(1.0%), which is the metabolite of MT in the liver unlike our moAb (0.009%, TABLE 1). These differences in crossreactivity could be attributable to polyclonal and monoclonal specificity. Pang et al.(5) found that selected amino acids, putative neurotransmitters or their derivatives which are abundant in biological

tissues had crossreactivities of less than 0.001%. Our data using the same compounds are in good agreement except that acetylcholine, which was not tested in their study, had crossreactivity of 0.001% which is relatively high among the compounds.

In order to investigate the effect of real samples, 0.1 ml of human serum was used in the RIA system; binding of [^{125}I]MT to antibody was inhibited as if about 30 ng of MT were contained in 0.1 ml of the serum. This inhibition must be due to interference by some compounds other than MT because human plasma levels of MT have been reported to be about 1 - 10 pg per ml in daytime and 40 - 80 pg per ml in night (9). Therefore we have to establish an extraction procedure to measure real MT levels in serum by consulting methods reported by other investigators such as (4,6).

The sensitivity of the present RIA with commercially pure MT is 50 pg, so it can be used to diagnose whether a man died in daytime or night with 1 or 2 ml of his serum. Pineal glands can also be utilized for the diagnosis, since pineal contents of MT in humans are much higher than the serum MT level(16,17). We will report the forensic application elsewhere in the near future.

This is believed to be the first reported success in preparing moAb against MT.

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REFERENCES

1. Wurtman, R. J. and Waldhauser, F., ed. Melatonin in Human. J. Neural Transm. 1986; Suppl. 21.

2. Waldhauser, F. and Dietzel, M. Daily and Annual Rhythms in Human Melatonin Secretion: Role in Puberty Control. Ann. N. Y. Acad. Sci. 1985; 453: 205-14.

3. Reiter, R.J. Normal Patterns of Melatonin Levels in the Pineal Gland and Body Fluids of Humans and Experimental Animals. J. Neural Transm. 1986; Suppl. 21: 35-54.

4. Arendt, J., Paunier, L. and Sizonenko, P. C. Melatonin Radioimmunoassay. J. Clin. Endocrinol. Metab. 1975; 40: 347-50.

5. Pang, S.F., Brown, G.M., Grota, L.J., Chambers, J.W. and Rodman, R.L. Determination of N-acetylserotonin and Melatonin Activities in the Pineal Gland, Retina, Harderian Gland, Brain and Serum of Rats and Chickens. Neuroendocrinology 1977; 23: 1-13.

6. Kawashima, K. and Nagakura, A. Improvement of Radioimmunoassay for Serum and Tissue Melatonin. J. Pharmacobiodyn. 1982; 5: s-26.

7. Brown, G.M., Grota, L.J., Pulido, O., Burns, T.G., Niles, L.P. and Snieckus, V. Application of immunologic techniques to the study of pineal indolealkylamines. In: Reiter, R.J., ed. Pineal Research Reviews, Vol.1. New York: Alan R. Liss, 1983: 207-46.

8. Arendt, J. Melatonin Assays in Body Fluids. J. Neural Transm. 1978; Suppl. 13: 265-78.

9. Arendt, J. Assay of Melatonin and its Metabolites: Results in Normal and Unusual Environments. J. Neural Transm. 1986; Suppl. 21: 11-33.

10. Grota, L.J. and Brown, G.M. Antibodies to Indolealkylamines: Serotonin and Melatonin. Biochem. Cell Biol. 1974; 52: 196-202.

11. Köhler, G. and Milstein, C. Continuous Cultures of Fused Cells Secreting Antibody of Predefined Specificity. Nature 1975; 256: 495-7.

12. Nagao, M., Takatori, T., Wu, B., Terazawa, K., Gotouda, H. and Akabane, H. Development and Characterization of Monoclonal Antibodies Reactive with Paraquat. J. Immunoassay 1989; 10: 1-17.

13. Ouchterlony, O. Antigen-antibody Reaction in Gels. APMIS 1949; 26: 507-15.

14. Yamaoka, A. and Takatori, T. Determination of Phenobarbital by Radioimmunoassay. J. Immunol. Methods 1979; 28: 51-7.

15. Lo, M. M. S., Tsong, T. Y., Conrad, M. K., Strittmatter, S.M., Hester, L.D. and Snyder, S.H. Monoclonal Antibody Production by Receptor-mediated Electrically Induced Cell Fusion. Nature 1984; 310: 792-4.

16. Beck, O., Borg, S. and Lundman, A. Concentration of 5-Methoxyindoles in the Human Pineal Gland. J. Neural Transm. 1982; 54: 111-6.

17. Stanley, M. and Brown, G.M. Melatonin Levels Are Reduced in the Pineal Glands of Suicide Victims. Psychopharmacol. Bull. 1988; 24: 484-8.