Preparation of Monoclonal Antibodies Reactive to a Hallucinogenic Drug, Psilocin

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The cultivation or trafficking of "Magic mushrooms," containing hallucinogenic psilocin and psilocybin, has been prohibited by the Narcotics and Psychotropics Control Law in Japan since 2002. To identify these mushrooms, we attempted to prepare the monoclonal antibody (mAb) reactive to these hallucinogens. As an antigen inducing an anti-psilocin mAb, *N*-{4-[3-(2-dimethylaminoethyl)indol-4-yl-oxy]butyl}succinamic acid was synthesized by modifying the 4-hydroxyl moiety of psilocin and coupled to a carrier protein of keyhole limpet hemocyanin. BALB/c mice were immunized five times with the antigen emulsified with an adjuvant, and their spleen cells were fused with mouse myeloma cells. We obtained several hybridoma cells producing mAbs reactive to psilocin, from which four clones, BA631, CA231, KA422, and MA332 with a higher production of anti-psilocin mAb were selected by limiting dilution. Isotype of CA231 and KA422 mAbs were IgG2a and that of BA631 and MA332 mAbs were IgG1. Enzyme immunoassay (EIA) using BA631 mAb, revealed that BA631 cross-reacted with psilocin and dimethyltryptamine, but not with the other indole derivatives such as psilocybin, 4-hydroxyindole, tryptamine, and tryptophan. Therefore, these antibodies could be used for the identification of magic mushrooms.

Key words —— psilocin, magic mushroom, enzyme immunoassay, monoclonal antibody

INTRODUCTION

The hallucinogenic agents, psilocin and psilocybin, are present in various mushrooms belonging to the genera of *Psilocybe*, *Panaeolina*, *Panaeolus*, *Copelandia*, *Conocybe*, *Gymnopilus*, *Stropharia*, and *Pluteus*,[1] which are sometimes called "magic mushrooms." Psilocin or psilocybin stimulates 5-hydroxytryptamine (5-HT_2) receptors, and then facilitates facial neuron excitation triggered by serotonin or norepinephrine.[2,3]

Magic mushrooms have been abused by youngsters in Japan. They can be easily obtained on the Internet or on the streets. The law, which prohibited the cultivation or trafficking of magic mushrooms, was enforced in Japan in June 2002. To regulate magic mushrooms, some rapid and precise methods to identify psilocin or psilocybin were required in any place. The detection methods in laboratories include TLC,[4] capillary zone electrophoresis,[5] gas chromatography,[6] GC-MS,[6] and mostly HPLC.[4,7,8] Furthermore, enzyme immunoassay (EIA) using a monoclonal antibody (mAb), is also useful in detecting illegal drugs in any place.[9] However, mAb against the hallucinogenic indole derivatives using EIA has not been established. Therefore, we prepared mAbs to psilocin for the identification of magic mushrooms in this study.

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MATERIALS AND METHODS

Chemicals —— Psilocin was synthesized from 4-hydroxyindole (4HI, Tokyokasei Co., Tokyo, Japan).[10] Bromobutylonitrile was purchased from Tokyokasei Co. Lithium aluminum hydride ($LiAlH_4$), bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), and horseradish peroxidase (HRP), were purchased from Wako Pure Chemical Co. (Osaka, Japan). Succinic anhydride

was purchased from Kokusan Chemical Co. (Tokyo, Japan). Cesium carbohydride, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl (EDC), and tetramethylbenzidine (TMB) were purchased from Sigma (MO, U.S.A.). All other chemicals used were of analytical grade.

Synthesis of *N*-{4-[3-(2-Dimethylaminoethyl)indol-4-yl-oxy]butyl}succinamic Acid (PSICOOH) ——— Cs_2CO_3 (16 mmol) was carefully added to psilocin (4 mmol) in acetonitrile (200 ml) with stirring. After 15 min at room temperature (rt), 4-bromobutylonitrile (6 mmol) was added to the mixture that was then stirred for 16 hr. After dilution with water (1 : 4), NaOH solution was added to the mixture, and the mixture was extracted with EtOAc. The extract was washed with brine, dried by Na_2SO_4, and evaporated to dryness under reduced pressure. The crude product was purified by silica gelcolumn chromatography using EtOAc-MeOH-28% aq. NH_4OH (90 : 10 : 1) as an eluent to obtain 4-[3-(2-dimethylaminoethyl)indol-4-yl-oxy]butylonitrile (PSICN, 574 mg, 52.4%, yellowish powder). The spectral data of the product are as follows. [^{1}H-NMR($CDCl_3$)δ: 2.2–2.3 (2H, m), 2.32 (6H, s), 2.6–2.7 (2H, m), 2.68 (2H, t, J = 7 Hz), 3.0–3.1 (2H, m), 4.20 (2H, t, J = 6 Hz), 6.46 (1H, dd, J = 8, 1 Hz), 6.9 (1H, m), 6.96 (1H, dd, J = 8, 1.5 Hz), 7.05 (1H, t, J = 7 Hz). ^{13}C-NMR($CDCl_3$)δ: 14.3 (t), 25.62 (t), 25.64 (t), 45.5 (q×2), 61.5 (t), 65.0 (t), 99.9 (d), 105.0 (d), 114.5 (s), 117.2 (s), 119.3 (s), 120.6 (d), 122.6 (d), 138.1 (s), 153.3 (s). FAB-MS *m/z*: 272([M+H]$^+$). HR-FAB-MS *m/z*: 272.1756 (Calcd for $C_{16}H_{22}N_3O$: 272.1763).]

$LiAlH_4$ (21 mmol) was then added to PSICN (2.1 mmol) in anhydrous tetrahydrofurane (THF) (300 ml) at 0°C with stirring, and the mixture was refluxed for 3 hr with stirring. To the reaction mixture, water saturated ether (100 ml) and 10% aq. Rochelle salt solution were added and mixture was extracted with EtOAc. The extract was washed with brine, dried using Na_2SO_4, and evaporated to dryness under reduced pressure. The crude product was purified by silica gel column chromatography using EtOAc-MeOH-28% aq. NH_4OH (90 : 10 : 1) as an eluent to obtain 4-[3-(2-dimethylaminoethyl)indol-4-yloxy]butylamine ($PSINH_2$, 353 mg, 61.0%, yellowish powder). The spectral data of products are as follows. [^{1}H-NMR($CDCl_3$)δ: 2.26 (2H, q, J = 7 Hz), 2.6 (4H, m), 2.66 (6H, s), 3.0–3.1 (2H, m), 3.29–3.33 (2H, m), 2.48 (2H, t, J = 7 Hz), 6.49 (1H, d, J = 8 Hz), 6.89 (1H, d, J = 2 Hz), 6.96 (1H, dd, J = 8, 1 Hz), 7.08 (1H, t, J = 8 Hz), 8.14 (1H, s). ^{13}C-NMR($CDCl_3$)δ: 14.5 (t), 22.2 (t), 25.3 (t), 51.9 (q×2), 66.0 (t×2), 66.3 (t), 100.3 (d), 105.1 (d), 112.3 (s), 121.0 (s), 122.9 (d), 138.1 (s), 153.0 (s). FAB-MS *m/z*: 276([M+H]$^+$). HR-FAB-MS *m/z*: 276.2082 (Calcd for $C_{16}H_{26}N_3O$: 276.2056).]

Finally, succinic anhydride (1.55 mmol) was added to $PSINH_2$ (1.29 mmol) in anhydrous THF (100 ml) with stirring. The mixture was incubated for 1 hr at rt with stirring and then evaporated to dryness under reduced pressure. The crude product was purified by silica gel column chromatography using EtOAc-MeOH-28% aq. NH_4OH (80 : 20 : 1) as an eluent to obtain *N*-{4-[3-(2-dimethylaminoethyl)indol-4-yloxy]butyl}succinamic acid (PSICOOH, 164 mg, 34.0%, yellowish powder). The spectral data of the product are as follows.

[^{1}H-NMR(CD_3OD)δ: 1.06–1.74 (2H, m), 1.88–1.93 (2H, m), 2.37–2.50 (6H, m), 2.85 (6H, s), 3.26–3.31 (2H, m), 3.38–3.41 (2H, m), 4.09 (2H, t, J = 7 Hz), 6.46 (1H, d, J = 8 Hz), 6.93 (1H, d, J = 8 Hz), 6.99 (1H, t, J = 8 Hz), 7.01 (1H, s). ^{13}C-NMR(CD_3OD)δ: 23.3 (t), 27.5 (t), 27.9 (t), 33.6 (t), 34.2 (t), 40.0 (t), 43.4 (q×2), 60.2 (t), 68.5 (t), 100.7 (d), 105.9 (d), 110.8 (s), 118.1 (s), 122.7 (d), 123.6 (d), 140.1 (s), 154.7 (s), 176.1 (s), 181.0 (s). FAB-MS *m/z*: 376 ([M+H]$^+$). HR-FAB-MS *m/z*: 376.2258 (Calcd for $C_{20}H_{30}N_3O$: 376.2237).]

Preparation of (PSICOOH)-Carrier Protein Conjugates ——— PSICOOH (25 mg) in H_2O (2 ml) was added to EDC (100 mg) in H_2O (2 ml), and incubated for 0.5 hr at rt with stirring. KLH, BSA, or HRP (25 mg) solution in H_2O (2 ml) to the mixture that then was added and incubated for 1 hr at rt and for 17 hr at 4°C with stirring. Each reaction product (PSI-HS, PSI-BSA, and PSI-HRP) was repeatedly dialyzed against PBS for a week at 4°C and stored in aliquots at –70°C until use.

Immunization with PSI-KLH and Preparation of Hybridoma Cells ——— Emulsion was prepared with equal volumes of PSI-KLH (0.1 mg, in PBS) and Titer-Max (Wako). Two female BALB/c mice (6 weeks, SLC, Sizuoka, Japan) were immunized i.p. five times with 0.2 ml of emulsion every 2 weeks. Three days after the final i.v. injection of the antigen without the adjuvant, spleen cells were prepared and fused with PAI mouse myeloma cells (6 : 1) using polyethylene glycol 4000 (Merck, Germany)[11] and cultured in the GIT medium (Wako) containing hypoxanthine, aminopterin and thymidine (HAT). Hybridoma cells producing the anti-psilocin antibody were subcloned four times by the limiting dilution method to ensure their monoclonal origin.

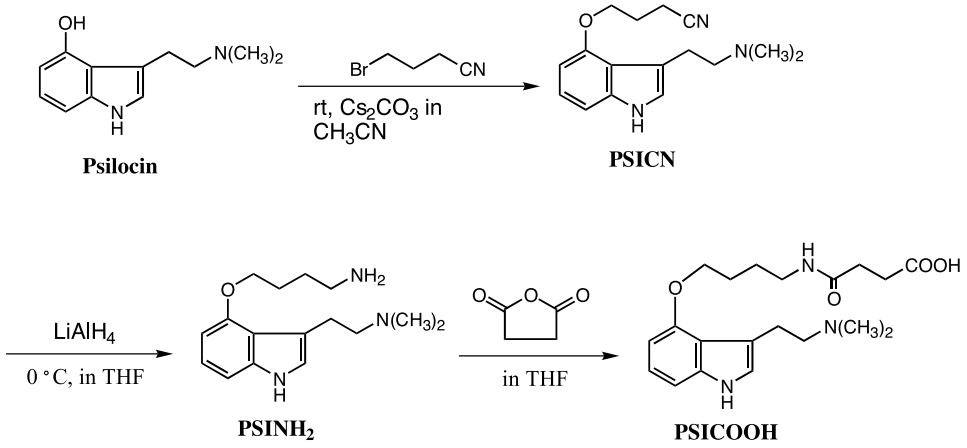

Fig. 1. Synthesis of *N*-{4-[3-(2-Dimethylaminoethyl)indol-4-yloxy]butyl}succinamic Acid

Purification of Monoclonal Antibodies and Analysis of their Isotypes ——— Conditioned media, in which hybridoma cells were cultured for 4 days, were dialyzed against 20 mM potassium phosphate buffer (pH 7.0) for 5 days. The antibies were purified from the conditioned medium using Protein G Sepharose (Amersham Bioscience, NJ, U.S.A.) and stored in aliquots at –70°C until use. The analyses of antibody isotypes were performed using a mouse immunoglobulin screening/isotyping kit (Zymed Lab., CA, U.S.A.). Absorption at 280 nm, determined in spectrophotometer, was used as protein concentration of purified antibody. Concentration (mg/ml) = (1.35 × A280).

ELISA for Titration of mAbs ——— The wells of a 96-well microtest plate (F96 Maxsorp Nunc-Immunoplate, Nunc, Denmark) were coated with 100 μl of PSI-BSA or BSA solution (1 μg/ml, 0.5 mM carbonate-bicarbonate buffer, pH 9.6) at 4°C overnight, blocked with 0.5% skimmed milk in 0.05% Tween 20-PBS (T-PBS) at rt for 3 hr, and washed with T-PBS. One hundred microliters of the antibody was added into the antigen-coated wells and incubated at rt for 90 min. After washing three times, 100 μl of HRP-conjugated anti-mouse IgGs (1 μg/ml T-PBS) was added to the wells and incubated for 60 min at rt. After washing three times, 150 μl of a color development reagent consisting of 1.0 mM TMB and 5.5 mM H_2O_2 was added into the wells and incubated at rt for 10 min. The reaction was stopped by an adding 50 μl of 1 M sulfuric acid. Absorbance at 450 nm was measured using a microplate reader (Model 3550, Biorad, CA, U.S.A.).

Competitive EIA for Determination of Psilocin ——— The wells of a 96-well microtest plate were coated with 100 μl of BA631-mAb (1 μg/ml of 0.5 mM carbonate-bicarbonate buffer, pH 9.6) at 4°C overnight, blocked with 0.5% skimmed milk in T-PBS at rt for 3 hr and washed with T-PBS.

Fifty microliters of PSI-HRP in PBS and 50 μl of psilocin in PBS (1–3333 μM) or indole derivatives in PBS (1–3333 μM) were mixed in microtubes. Then, they were transferred into the antibody-coated wells and incubated at rt for 0.5 hr.

After washings, 150 μl of the color development reagent was added to the wells and incubated at rt for 10 min. The reaction was stopped by adding 50 μl of 1 M sulfuric acid. Absorbance at 450 nm was measured using a microplate reader. Percent cross-reactivity was calculated as follows: If x is the concentration of an indole derivative in μM required to displace 50% of PSI-HRP bound to the antibody and y is that of psilocin in μM required to displace 50% of PSI-HRP bound to the antibody, then percent cross-reactivity is $(x/y) \times 100$.

RESULTS AND DISCUSSION

Although psilocin derivatives with an alkyl spacer in position 1 of the indole ring was already synthesized,[12] the antibody to psilocin has not been raised. As an antigen inducing anti-psilocin mAb, we synthesized a psilocin derivative with an alkyl spacer at the phenol hydroxyl group in position 4 of the indole ring (hapten, Fig. 1) and coupled the derivative to a carrier protein of hemocyanin.

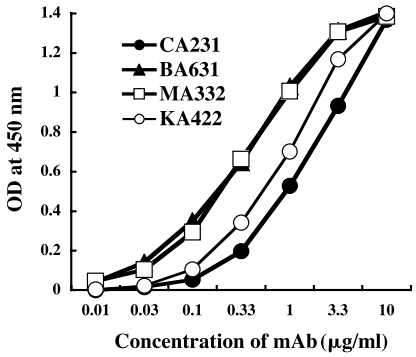

Fig. 2. Reactivity of Monoclonal Antibodies Produced by PSI-BA631, CA231, KA422, and MA332 Clones to PSI-BSA

Using PSI-BSA coated plate, the titration curve of purified antibodies was measured.

Table 1. Subtype of the Anti-Psilocin mAbs Produced by Clones

Clone	Subtype	Light chain
BA631	IgG2a	lambda
CA231	IgG1	lambda
KA422	IgG2a	lambda
MA332	IgG1	lambda

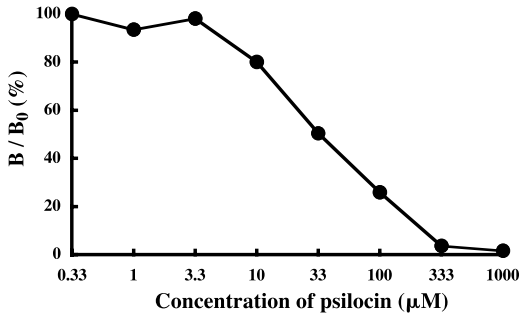

Fig. 3. Dose Response Curve of Psilocin Detected by EIA Using BA631-mAb

Dose response curve (%) of PSI-HRP binding with various concentration of psilocin (B) as a rate of that bound using buffer (B_0).

Hybridoma cells were obtained by fusion between lymphocytes from mice hyperimmunized with the hapten-carrier and mouse myeloma cells. Finally, four clones, namely CA231, BA631, MA332, and KA422, secreting mAb reactive with PSI-BSA, but not with BSA, were selected from the hybridoma cell clones. The titration curves of IgG fractions purified from the culture supernatants of these clones are shown in Fig, 2. BA631-mAb and MA332-mAb were more reactive to PSI-BSA than KA422-mAb and CA231-mAb. The isotypes of CA231 and KA422 mAbs were IgG2a with the lambda light chain, and the isotypes of BA631 and MA332 mAbs were IgG1 with the lambda light chain (Table 1).

The dose response standard curve of psilocin is shown in Fig. 3. The range of psilocin detection by EIA using BA631-mAb was between 3.3 μM (0.67 μg/ml) and 333 μM (67.4 μg/ml) psilocin.

The specificity of BA631-mAb was determined by percent cross-reactivity with several indole derivatives (Fig. 4), as the ratio of each compounds in terms of the concentration required for 50% inhibition of PSI-HRP binding to the antibody coated plate. The antibody cross-reacted with dimethyltryptamine by 148% (Table 2). However, it did not cross-react with the other compounds, namely, psilocybin, tryptamine, 4-hydroxyindole and tryptophan even at 3.33 mM.

These findings suggested the antibody well recognized the dimethylaminoethyl moiety. The possible reason that antibody did not recognize psilocybin with the phophate moiety, is that the phosphate moiety at position 4 of the indole ring may interfere with the binding with the antibody.

Thus far, some color development test kits for psilocybin and psilocin have been commercially available. However, their principle was based on Ehrlich reaction and these kits showed false positive for the other indole compounds in mushrooms.

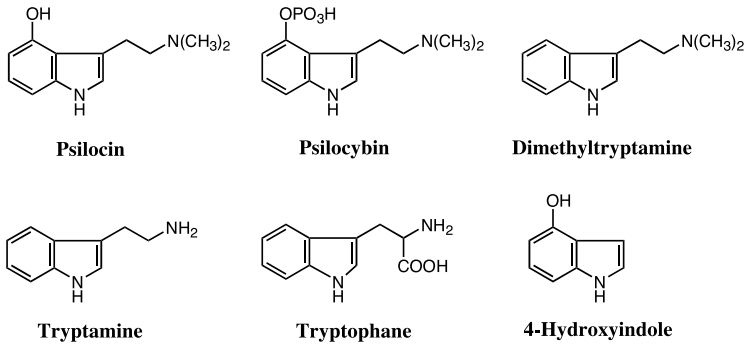

Fig. 4. Structures of Various Indole Derivatives

Table 2. Percent Cross-Reactivity of BA631-mAb with Indole Derivatives

Derivatives	Cross-reactivity (%)
Psilocin	100.0
Psilocybin	< 1.0
Dimethyltryptamine	147.8
Tryptamine	< 1.0
Tryptophan	< 1.0
4-Hydroxyindole	< 1.0

Percent cross-reactivity was calculated as follows: If x is the concentration of an indole devative in μM required to displace 50% of PSI-HRP bound to the antibody and y is that of psilocin in μM required to displace 50% of PSI-HRP bound to the antibody, then percent cross-reactivity is $(x/y) \times 100$.

In the present study, we established the mAb reactive to a psilocin. However, it did not cross-react with psilocybin. As it is known that psilocin is detected in a variety of hallucinogenic mushrooms,[13] the affinity of these antibodies are sufficient for the identification of magic mushrooms. To be able to identify magic mushrooms in places other than laboratories, we are now preparing a handy and precise immunochromatography kit for detecting of magic mushrooms.

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