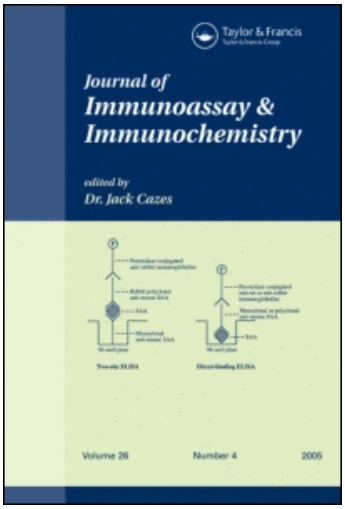

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

Development of Monoclonal Antibodies Reactive With Methamphetamine Raised Against A new Antigen

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To cite this Article Terazawa, Koici , Ji, Liying , Takatori, Takehiko , Aoki, Kimiko , Hirose, Yasuko and Kuroiwa, Yukio(1991) 'Development of Monoclonal Antibodies Reactive With Methamphetamine Raised Against A new Antigen', Journal of Immunoassay and Immunochemistry, 12: 2, 277 — 291
To link to this Article: DOI: 10.1080/01971529108055072
URL: http://dx.doi.org/10.1080/01971529108055072

JOURNAL OF IMMUNOASSAY, 12(2), 277-292 (1991)

DEVELOPMENT OF MONOCLONAL ANTIBODIES REACTIVE WITH METHAMPHETAMINE RAISED AGAINST A NEW ANTIGEN

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ABSTRACT

Monoclonal antibodies (McAbs) specific to methamphetamine (MA) were produced using p-amino MA coupled to bovine serum albumin (BSA) with glutaraldehyde (GA) as an immunogen and with conventional hybridoma techniques. Hybridoma clones secreting the McAbs were selected by an enzyme-linked immunosorbent assay (ELISA) system using both the above conjugate and BSA modified with GA as screening antigens. In the ELISA system were used avidin and biotinyl-alkaline phosphatase which converts nicotinamide adenine dinucleotide phosphate (NADP) into NAD. The final enzyme activity was determined using diformazan of nitroblue tetrazolium formed together with the NAD produced, alcohol dehydrogenase and phenazine methosulfate. The McAbs from 9 clones were characterized by a crossreactity test using the ELISA. The McAbs recognized MA (100 %), methoxyphenamine (8.0 %), ephedrine (2.3 %), but did not react with methylephedrine, amphetamine, OH-amphetamine, dimethylamphetamine, β-phenylethylamine, norephedrine, phentermine and ranitidine. An inhibition curve for MA was obtained in the range of 0.75 to 50 ng. (KEY WORDS: Methamphetamine, p-Amino-methamphetamine, Monoclonal antibdy, Immunoassay, Enzyme-linked immunosorbent assay.)

INTRODUCTION

Immunoassays for methamphetamine (MA) have been established by preparing antibodies raised against the hapten-carrier antigens (1-8). The immunogens have been synthesized by modifying two parts of MA molecule, i.e., amino group and p-position of the aromatic ring.

To the amino group of MA the following spacers were attached; $-CH_2COOH$ (1), $-(CH_2)_3-COOH$ (3) and $-(CH_2)_4NH_2$(4-7,9); the carboxy or amino group introduced was combined with carrier molecules. However, polyclonal antibodies (PcAbs) raised against the above immunogens crossreacted with not only MA-related drugs but their metabolites (2-6);methylephedrine (2-20.8 % crossreactivity), ephedrine (0.5-13 %), amphetamine (0.6-6.6 %), norephedrine (0.1 and 2 %) and methoxyphenamine (0.05-2.2 %). On the other hand, monoclonal antibodies (McAbs) were prepared recently by immunizing a conjugate of MA-$(CH_2)_4$-NHCO-human IgG (7), but one of 15 clones obtained had lower crossreactivity with almost all of the above compounds than PcAbs, and still had undesirable reactivity with methylephedrine (1.6 %).

To solve the problem, our coworkers prepared a new PcAb (8). They introduced amino group at p-position of

the aromatic ring of MA (p-aminoMA) and coupled it to bovine serum albumin by glutaraldehyde method (p-amino-MA-GA-BSA). The produced PcAb did not crossreact with methylephedrine but did weakly with methoxyphenamine (7.2 %), ephedrine (0.8 %) and amphetamine (0.3 %).

Since it is generally known that McAbs give us higher specificity than PcAbs and that the former also are superior in an immunohistochemical study to the latter, in this paper we deal with the production and characterization of McAbs raised against the p-aminoMA-GA-BSA antigen.

MATERIALS AND METHODS

Preparation of Antigen Conjugates

(i) An immunogen, p-aminoMA-GA-BSA, was prepared by coupling p-aminoMA to BSA by condensation with GA followed by reduction with sodium borohydride (FIGURE 1) as previously described (8,10).

(ii) BSA was modified with GA (BSA-GA) for the McAb screening antigen of an enzyme-linked immunosorbent assay (ELISA) as described above.

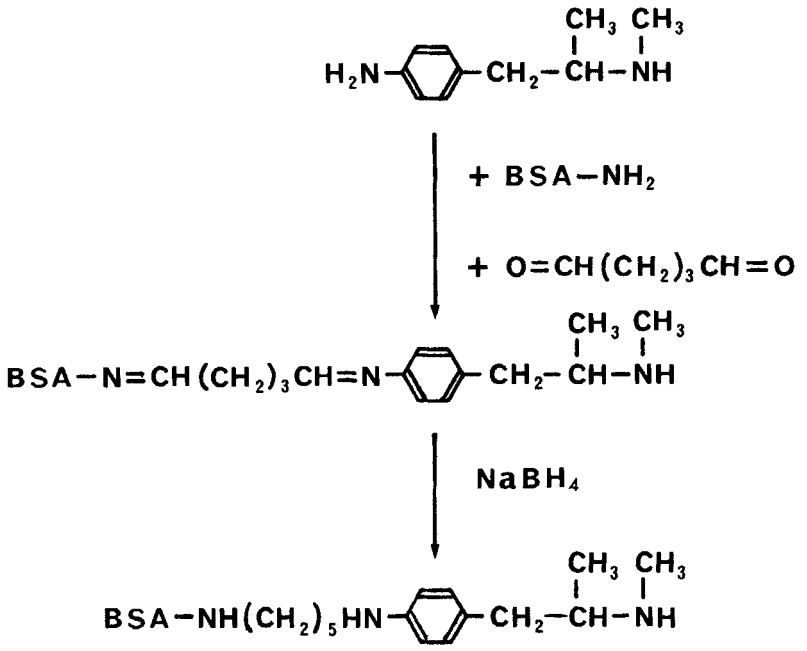


FIGURE 1. Synthesis of an antigen by conjugation of p-aminomethamphetamine and bovine serum albumin through glutaraldehyde followed by reduction.

Production of McAbs

A female six-week old BALB/C mouse was first immunized i.p. with 0.25 mg of p-aminoMA-GA-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 of the immunogen 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 McAbs were selected by the ELISA system described below using the p-aminoMA-GA-BSA and BSA-GA as screening antigens. Hybridoma cells contained in wells in which culture supernatants were positive to p-aminoMA-GA-BSA but negative to BSA-GA, were subcloned twice by a limiting dilution method to ensure their monoclonal origins.

Isotype analyses were performed using a mouse monoclonal typing kit (MMT-RC 1, Serotec Ltd., U.K.). Culture supernatant was used as a source of antibody.

ELISA

All procedures were carried out at room temperature except otherwise mentioned. One hundred µl of 10 mM sodium carbonate buffer (pH 9.6) containing 1 µg of p-aminoMA-GA-BSA were added to each well of 96 flat-bottomed well plate (Falcon 3915, Becton Dickinson & Co., U.S.A.) and incubated overnight at 4 °C. The coated wells were then washed three times with PBS containing 0.05 % Tween 20 (PBS-Tween). In order to block vacant sites on the well surface, 400 µl of 1 % gelatin in PBS-Tween (1%G-PBS) were added to each well and the

plate incubated for 30 min. To each well were added 100 µl of culture supernatant by the McAb screening, alternatively 50 µl of either MA or its related compound solution in 0.1%G-PBS followed by the addition of 50 µl of diluted McAbs in 0.1%G-PBS by preparing calibration curve or crossreactivity test. The plate was allowed to stand for 2 h. The wells were then washed three times with 0.1%G-PBS. Subsequently, 100 µl of biotinylated horse anti-mouse IgG (H+L) (Vector, U. S. A., 6 µg protein/ml in 0.1%G-PBS) were added to the wells with 0.01 M Tris-buffered saline containing 0.05 % Tween 20, pH 7.4 (TBS) and 100 µl of avidin-biotinylated alkaline phosphatase TBS solution containing 0.5 µl of each of A and B reagents of a Standard Vectastain ABC Kit (Vector, U.S.A.) were added to the wells, and incubated for 30 min. After washing with TBS three times, 100 µl of 0.05 M diethanolamine buffer (pH 9.5) containing 0.2 mM nicotinamide adenine dinucleotide phosphate (NADP, Boehringer Mannheim GmbH, F.R.G.) were added to the wells. After incubating for 20 min, 100 µl of 0.05 M phosphate buffer (pH 7.0) containing 3 % (w/v) ethanol, 1 mM phenazine methosulfate (PMS, Wako Pure Chem. Ind. Ltd., Japan), 1 mM nitro blue tetrazolium (NBT, Wako Pure Chem. Ind. Ltd., Japan), and 17 µg of alcohol dehydrogenase (ADH) from yeast (280 U/mg powder, Oriental yeast, Japan). After a further incu-

bation for 10 min, the enzymic reaction was stopped by the addition of 50 µl of 0.2 M sulfuric acid. The absorbance at 660 nm in each well was measured on an MTP-22 microplate photometer (Corona Electric Co., Japan).

RESULTS AND DISCUSSION

Nine clones of hybridoma, all secreting McAbs specific to p-aminoMA-GA-BSA but not reacting with BSA-GA, were selected. The specificity of each antibody was characterized by the crossreaction test. Crossreactivity of an MA analogue was calculated as (A ÷ B)× 100 (%), where A is the weight of MA which reduced the absorbance at "0" (1.1 in FIGURE 2) by half (to 0.55) and B is that of an MA analogue. The nine McAbs showed almost the same features and representative results are shown in TABLE 1. The immunoglobulin isotype of each McAb was tested by a reverse passive haemagglutination method using a commercial kit (Serotec Ltd., U.K.) and all were identified as IgG_1.

The clone secreting the highest level of McAb in culture medium was used to examine sensitivity of the assay, the culture supernatant being used as a source of McAb. The inhibition curve for MA in the range of 0.75 to 50 ng was obtained by using a 1:100 dilution of

TABLE 1

Crossreactivity of the Present Monoclonal Antibody with Methamphetamine and its Analogues

Analogues	% Crossreactivity
Methamphetamine	100
OH-methamphetamine	100
Methoxyphenamine	8.0
Ephedrine	2.3
Norephedrine	<0.01
Methylephedrine	<0.01
Amphetamine	<0.01
OH-amphetamine	<0.01
Dimethylamphetamine	<0.01
Phentermine	<0.01
β-Phenylethylamine	<0.01
Ranitidine	0.012

the supernatant, with 10 ng of MA giving 50 % inhibition (FIGURE 2). The value of 0.75 ng of MA corresponding to 10 % inhibition as the detection limit was taken (12).

In order to make the ELISA more sensitive than the conventional avidin-biotin complex (ABC) colour development method (13), an enzyme-amplification method described by Stanley et al. (14) was used with a slight

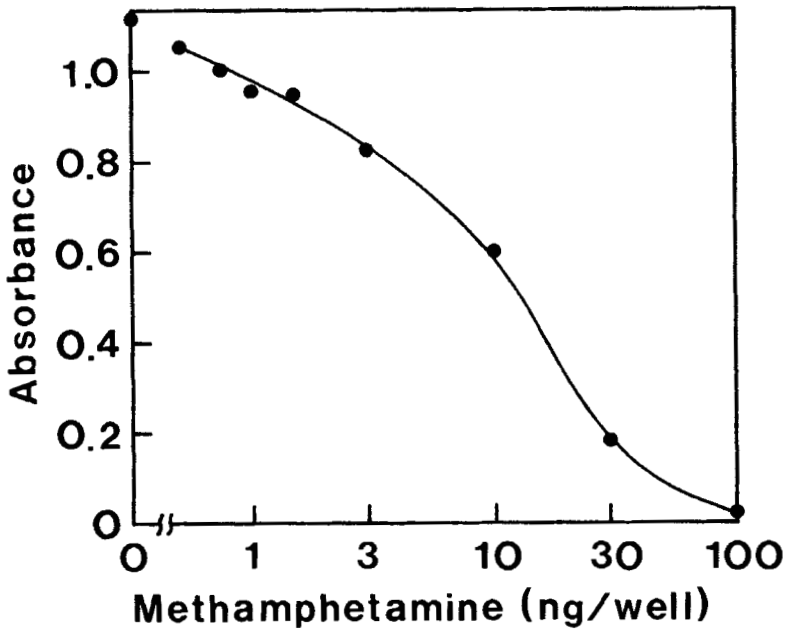


FIGURE 2. Calibration curve for methamphetamine by an enzyme-linked immunosorbent assay using a monoclonal antibody raised against a new antigen.

modification. In the method, the alkaline phosphatase labelled to biotin first dephosphorylates NADP in the mixture, the NAD formed is involved in a member of an NAD-specific redox cycle, and a formazan dye is finally yielded. As members of the redox cycle were used ethanol, ADH, iodonitrotetrazolium violet (INT, Research Organics Inc., U.S.A.) and diaphorase from microorganisms (Boehringer Mannheim GmbH, F.R.G.) as originally reported (14). But absorbance of a reagent

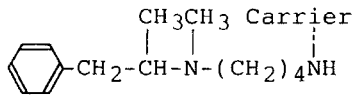
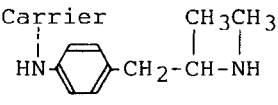
blank was constantly very high (about 1.0), which made indistinguishable the decrease in absorbance by MA inhibition. Therefore we changed the two members in the redox cycle, i.e., INT and diaphorase to PMS and NBT. The resultant absorbance of the reagent blank was satisfactory, and the sensitivity became four times higher than that of the conventional ABC method using phenylphosphoric acid, 4-aminoantipyrine and potassium ferricyanide (13). The sensitivity of ELISA for MA is not less sensitive than that of previous investigators, i.e., 0.5-1 ng/well (6,7).

Comparing the present McAb with the previous PcAb (8), both were raised against the same antigen, and the crossreactivity of the McAb with methylephedrine and amphetamine was considerably lower than that of the PcAb but that with ephedrine was slightly higher (TABLE 2 and FIGURE 3).

We note that the McAb raised against N-(4-aminobutyl)MA-carrier antigen by Usagawa et al. (7) crossreacted weakly with methylephedrine and ephedrine when compared with the PcAbs prepared previously with the similar antigen (4,6). But their McAb still crossreacted not only with methylephedrine (1.6 % crossreactivity), but also with amphetamine (1.9 %), dimethylamphetamine (150 %) and phentermine (0.4 %). On the contrary, our McAb did not crossreact (<0.01 %)

TABLE 2

Comparison of % Crossreactivities with Methamphetamine and its Analogues of Polyclonal (Pc) and Monoclonal (Mc) Antibodies Raised against Two Sorts of Methamphetamine-Carrier Antigen

					
	PcAbs		McAb	PcAb	McAb
Analogues	(4)	(6)	(7)	(8)	Ours
Methamphetamine	100	100	100	100	100
Methylephedrine	20.8	20	1.6	0.03	<0.01
Amphetamine	4.6	6.6	1.9	0.2	<0.01
Methoxyphenamine	1.2	1	0.2	7.2	8.0
Ephedrine	4.7	13	0.1	0.8	2.3
β-Phenylethylamine	-	-	-	0.01	<0.01
OH-methamphetamine	0.2	0.2	1.4	135	100
OH-ephedrine	<0.1	<0.1	0.3	1.69	-
Dimethylamphetamine	-	266	150	-	<0.01
OH-amphetamine	-	<0.1	<0.1	-	<0.01
Norephedrine	-	2	<0.1	-	<0.01
Phentermine	1.3	6.1	0.4	-	<0.01

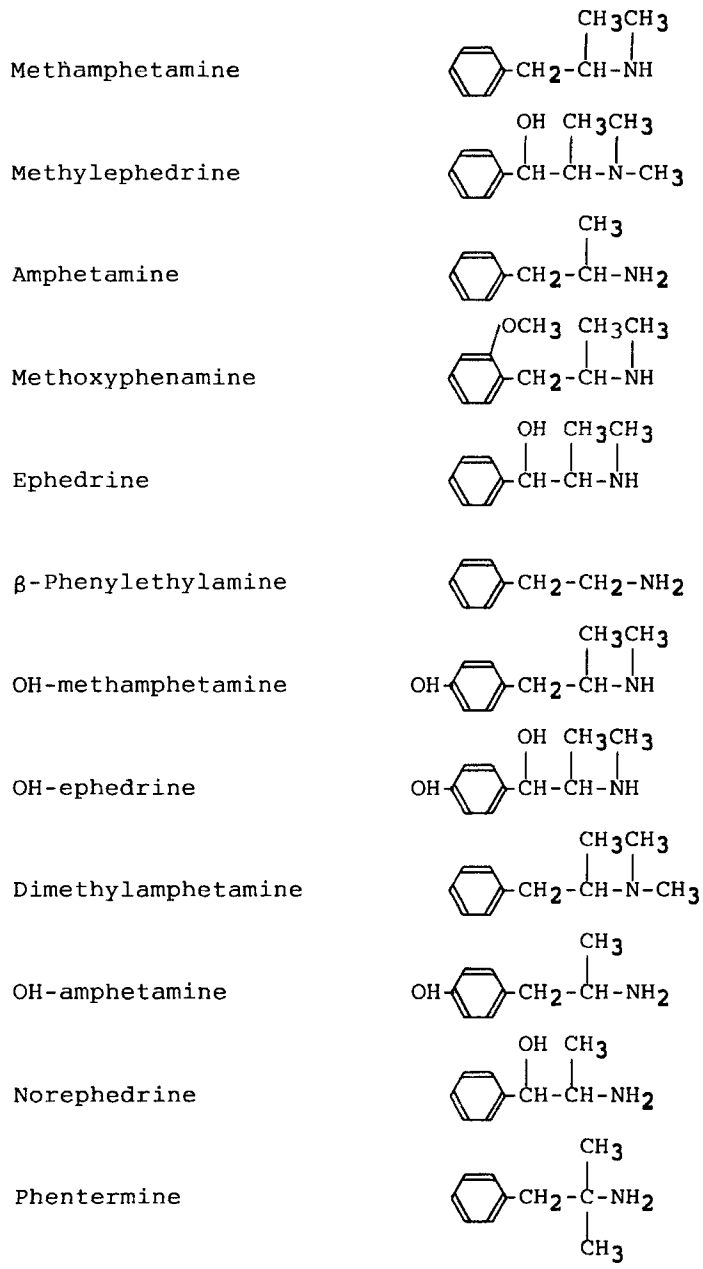


FIGURE 3. Structural formuli of methamphetamine and its analogues listed in TABLE 2.

with these four compounds, although reacted strongly with OH-MA (100 %), which is a metabolite of MA (15). In this study, the crossreaction test with OH-ephedrine and benzathine was not given. However, it is estimated that our McAb would crossreact weakly with OH-ephedrine because it does not recognize p-hydroxy group of the aromatic ring. Our McAb also would not crossreact with benzathine (7,16) because it recognizes whether or not methyl group at the carbon-2 position as indicated by ß-phenylethylamine exists. Our McAb did not crossreact with ranitidine (0.012 % crossreactivity), a drug reported to give false positive with EMIT screening test which uses an McAb (17).

Usagawa's and our McAbs also had weak crossreactivities with methoxyphenamine (0.2 and 8.0 %) and ephedrine (0.1 and 2.3 %, respectively). Therefore, ephedrine, a metabolite of methylephedrine (18), could give a false positive even by our ELISA at the screening for MA in samples.

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