# Production and Characterization of Monoclonal Antibody That Simultaneously Recognizes Methamphetamine and Its Major Metabolite

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**A series of monoclonal antibodies (mAbs) that react with methamphetamine–bovine serum albumin (MA-BSA) were established by intrasplenic immunization method. Among established 36 clones, two typical mAbs, designated NK-1 and NK-2, were described. The inhibition assay of enzyme-linked immunosorbent assay (ELISA) analysis using methamphetamine analogs indicated that NK-1 showed considerable reactivity not only MA-BSA but also methamphetamine and its major metabolite, *para*-hydroxymethamphetamine (*p*-hydroxymethamphetamine). The cross-reactivity between NK-1 and the methamphetamine analogs with modified alkyl side chain, indicates that methyl groups of $R_5$ and $R_7$ in the methamphetamine molecules are important for the maximum affinity. The length of alkyl side chain on methamphetamine significantly affected the binding affinity of NK-1. The results may suggest that NK-1 will recognize not only methamphetamine but also the bridge part of the methamphetamine that binds the methamphetamine molecules to a carrier protein.**

**Keywords** monoclonal antibody; methamphetamine; *p*-hydroxymethamphetamine

## Introduction

Methamphetamine is one of the most potent sympathomimetic amines with respect to stimulation of the central nerve system (CNS). It stimulates the medullary respiratory center, lessens the degree of central depression caused by various drugs, and produces other signs of stimulation of the CNS.[1] Recently, its increasing frequency of abuse and narcotic effect have been resulted in an urgent need for a rapid, and reliable screening method for its detection in human urine.

Unlike gas-chromatographic and infrared spectrophotometric methods, radioimmunoassay for methamphetamine are highly practical in that biological specimens need no solvent extraction. However, a radioimmunoassay requiring a well-equipped laboratory is not suitable for forensic science routine. Takemi *et al.* reported solid-phase micro ELISA for methamphetamine using horseradish peroxidase (HRP)-labeled methamphetamine.[2] To better analyze the urinary metabolites of methamphetamine, first of all, high affinity monoclonal antibody (mAb) which has cross-reactivity with methamphetamine and its metabolites was needed. It was generally accepted that methamphetamine has low molecular weight and poor antigenicity. Therefore, to make mAb with the usual immunization protocol was very difficult in our experience. In this report, we directly immunized methamphetamine–bovine serum albumin (MA-BSA) into the spleen of Balb/c mice and effectively established mAbs which simultaneously recognized methamphetamine and its major metabolite.

### Materials and Methods

**Animal** Asceptically fed female Balb/c mice (6 to 8 weeks, Korea Research Institute of Chemical Technology, Taejeon, Korea) were used and were fed standard rodent chow and water *ad libitum* before use in this study.

**Materials** Methamphetamine and its analogs were kindly provided by Doping Control Center, KIST, Seoul, Korea. Polyvinyl chloride microtiter plates (Immulon 2) were from Dynatech Laboratories, Inc. RPMI, hypoxanthine, thymidine and aminopterin were purchased from Sigma Chemical (St. Louis, MO.). Polyethylene glycol 4000 was bought from Merck (Darmastadt, F.R.G.). Protein A-Sepharose CL-4B was obtained from Pharmacia (Uppsala, Sweden). Fetal bovine serum was obtained from Gibco Laboratories (Grand Island, N.Y.).

**Preparation of MA-BSA Conjugate** Methamphetamine was activated to *N*-(4-aminobutyl)methamphetamine using *N*-(4-bromobutyl)phthalimide and hydrazine hydrate by the method of Iwasaki.[3] The activated $MA-NH_2$ was coupled to BSA using bifunctional agent, EDAC [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide] and MA-BSA conjugate was obtained, after the polymerized conjugation of MA-BSA was removed by Sephacryl S-200 chromatography.

**Production of mAb against Methamphetamine** *Salmonella minesota*, strain R595 was kindly provided by Dr. M. Umeda (the pharmaceutical Science, the University of Tokyo). The *Salmonella* were coated with MA-BSA and the suspension containing 10 $\mu$g of the methamphetamine and 50 $\mu$g of *Salmonella* in 0.2 ml of 20 mM phosphate buffer (pH 7.4) and 150 mM NaCl (phosphate buffered saline, PBS) were prepared as described previously.[4,5] Immunization of mice was performed by the intrasplenic injection as described previously.[6,7] Three days after the second intrasplenic injection (2 weeks interval), $10^8$ spleen cells were fused with $1.5 \times 10^7$ Sp2/0-Ag14 mouse myeloma cells by treatment with 0.5 ml 50% polyethylene glycol in RPMI (Roswell Park Memorial Institute) 1640. Two weeks after the fusion, the supernatants of hybridoma were tested for production of anti-MA-BSA antibodies of ELISA (enzyme-linked immunosorbent assay). The positive cells were further tested by inhibition of ELISA. The mAbs in the ascites fluid were purified by 50% ammonium sulfate precipitation followed by affinity chromatography on a Protein A-Sepharose column.[8] The heavy chain classes of the mAbs were determined by using a double immunodiffusion test.

**Binding of the mAb to MA-BSA** The binding of the mAb to MA-BSA was measured by ELISA.[9] In brief, the wells of the microtiter plates were coated with 50 $\mu$l of the MA-BSA antigen in coating buffer (pH 9.5). The wells were blocked with a solution containing BSA (30 mg/ml) and incubated with hybridoma supernatants. The antibody bound was detected by biotinylated anti-mouse Igs (ZYMED Laboratories, San Francisco, CA) followed by incubation with peroxidase-conjugated streptavidin (ZYMED Laboratories). Optical density at 490 nm was determined by the addition of *o*-phenylenediamine substrate in ELISA reader (Titertek Multiskan MCC/340, Labsystems, Finland). Throughout this study we used PBS containing 0.05% Tween 20.

**Inhibition Assay of ELISA** Inhibition of the binding of anti-methamphetamine antibodies by water soluble analogs was performed as follows: Fifty $\mu$l of the mixture containing mAb and the various analogs were preincubated for 1 h at room temperature and the mixture was transferred to the microtiter wells coated with MA-BSA. The amounts of antibody bound to the plate were measured as described above. All methamphetamine analogs containing *p*-hydroxymethamphetamine were

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dissolved in PBS and used for the experiment.

## Results

**Specificities of mAbs against MA-BSA** In our preliminary experiments, the binding of the specific mAbs to methamphetamine was analyzed by direct binding of the mAb to the MA-BSA coated on the microtiter plates (ELISA) (Fig. 1). And then, further analyses using inhibition assay of the ELISA by aqueous methamphetamine or other water soluble analogs were performed. Among 36 clones obtained, results obtained two typical mAbs named NK-1 ($IgG_1$) and NK-2 ($IgG_1$), are described in this paper. Both mAbs had high affinity to MA-BSA and no cross reaction with carrier protein (BSA).

**Inhibition Assay of ELISA** In order to further analyze the interaction between the mAb and methamphetamine, an inhibition analysis of the ELISA by methamphetamine analogs was performed. NK-1 showed considerable reactivity with methamphetamine and its major metabolite (*p*-hydroxymethamphetamine), and 50% inhibition of the binding of NK-1 was observed at 40 nmol of methamphetamine and 45 nmol of *p*-hydroxymethamphetamine, and only weak inhibition was observed with other metabolite, amphetamine (Fig. 2). However, other methamphetamine analogs failed to inhibit the binding of NK-1. On the other hands, in contrast to NK-1, NK-2 could not react with methamphetamine and its metabolites (data not shown). The detailed ELISA inhibition assay results were shown in Table 1. Phenyl ring modification of methamphetamine to methoxy group (methoxyphenamine) was approximately observed with 100% inhibition. However, the analyses using other methamphetamine analogs with modified alkyl side chains such as epinephrine, amphetamine and norephedrine were observed no appreciable inhibition. Although *p*-hydroxyephedrine showed no inhibition, these results suggested that methyl groups of $R_5$ and $R_7$ in the methamphetamine molecules were important for the binding of NK-1. In order to obtain further information on the interaction between anti-methamphetamine mAb and al-

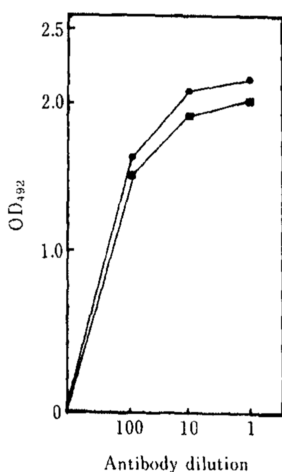


Fig. 1. Reactivity of NK-1 and NK-2 with MA-BSA

Microtiter plates were coated with 2 µg of MA-BSA. NK-1 (●) and NK-2 (■) were detected with biotinylated anti-mouse Igs and streptavidin-conjugated peroxidase.

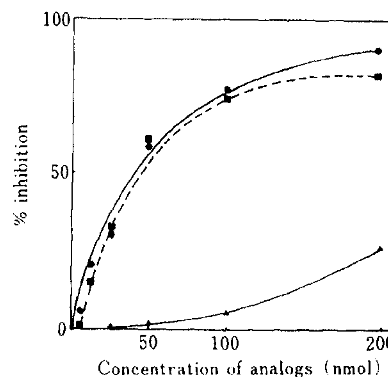


Fig. 2. Inhibition Assay of ELISA of NK-1 by Methamphetamine, Amphetamine and *p*-Hydroxymethamphetamine

NK-1 was preincubated with water soluble methamphetamine (●), amphetamine (▲) and *p*-hydroxymethamphetamine (■), and the mixtures were transferred to the microtiter wells coated with MA-BSA. After incubation, the mAb bound was detected with biotinylated anti-mouse Igs and streptavidin-conjugated peroxidase.

TABLE 1. Cross-Reaction of NK-1 with Methamphetamine and Other Analogs

General structure (table heading): a benzene ring with $R_1$, $R_2$ and $R_3$ on the ring and the side chain $-CH(R_4)-CH(R_5)-N(R_6)-R_7$

| Compounds | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ | $R_6$ | $R_7$ | % cross reactivity (50% inhibition) |
|---|---|---|---|---|---|---|---|---|
| Methamphetamine | H | H | H | H | $CH_3$ | H | $CH_3$ | 100 |
| Methoxyphenamine | $CH_3O$ | H | H | H | $CH_3$ | H | $CH_3$ | 100 |
| *p*-Hydroxy methamphetamine | H | OH | H | H | $CH_3$ | H | $CH_3$ | 85.5 |
| *p*-Hydroxy ephedrine | H | OH | H | OH | $CH_3$ | H | $CH_3$ | 9.5 |
| Epinephrine | H | OH | OH | OH | H | H | $CH_3$ | 20.0 |
| Amphetamine | H | H | H | H | $CH_3$ | H | H | 2.2 |
| Norephedrine | H | H | H | OH | $CH_3$ | H | H | 9 |
| Ephedrine | H | H | H | OH | $CH_3$ | H | $CH_3$ | 26.0 |
| *dl*-Methylephedrine | H | H | H | OH | $CH_3$ | $CH_3$ | $CH_3$ | 52.2 |
| Ethylephedrine | H | H | H | OH | $CH_3$ | $CH_3$ | $CH_2CH_3$ | 15.4 |

NK-1 was preincubated with methamphetamine or various analogs and the mixtures were transferred to the microtiter wells coated with MA-BSA. After incubation, the mAb bound was detected as described in Fig. 2.

$$\%\ \text{cross reactivity} = \frac{50\%\ \text{inhibition concentration of analogs to NK-1 binding}}{50\%\ \text{inhibition concentration of MA to NK-1 binding}} \times 100$$

kyl moieties of methamphetamine molecule, methyl group of $R_7$ substituted with ethylfunctional group (ethylephedrine) was studied. Ethylephedrine did not inhibit the binding of NK-1 to MA-BSA.

## Discussion

It is well known that methamphetamine is metabolized to *p*-hydroxymethamphetamine and amphetamine in the liver microsomal system, $P_{450}$. Among two metabolites. *p*-hydroxymethamphetamine is known to the major metabolite in human. Several laboratories have attempted to prepare antibodies to methamphetamine metabolites and the degree of success has been relatively low when compared with antisera prepared for other drugs. Very recently. Iwasaki reported that polyclonal antibody against *N*-(4-aminobutyl)-methamphetamine showed cross reactivity with methamphetamine, but little cross reactivity with amphetamine and *p*-hydroxymethamphetamine by the soluble antibody enzyme immunoassay system with alkaline phosphatase.[3] In this respect, the antibody which shows fine affinity against *p*-hydroxymethamphetamine is necessary to detect methamphetamine in human blood and urine.

Intrasplenic immunization technique, in which the antigen is deposited into the spleen tissue, is appropriate when only small amount of immunogen is available.[10] Although the effective activation of the splenic B cells was achieved by injection of the antigen directly into the spleen, this method was not generalized. Actually, we tried to produce anti-methamphetamine mAb using the usual immunization protocol several times, we failed to. However, we established a series of mAbs that react with methamphetamine by intrasplenic immunization method. NK-1 was highly specific to MA-BSA and cross-reacted with soluble methamphetamine. In the analysis using synthetic methamphetamine analogs, methoxyphenamine = methamphetamine > *p*-hydroxymethamphetamine ≫ amphetamine = norephedrine, indicating the methyl groups of $R_5$ and $R_7$ in methamphetamine may play an important role in the antigen–antibody interaction. Although only one methylene increased, the inhibition of NK-1 binding significantly decreased with the increasing acyl chain length of methamphetamine molecule. Additionally, NK-1 recognized not only methamphetamine but also the bridge part of the methamphetamine immunogen that bound the methamphetamine molecules to a carrier protein.

The mAb that specifically recognizes a certain antigen (ligand) may not only provide valuable information about ligand receptor (protein) interaction but may also represent a structural temperature for the production of anti-idiotypic antibody that could recognize the cross-reactive structures shares between the antibody and the actual receptor molecules.[11-13] Although further detailed reactivity profile and idiotypic analyses of the anti-methamphetamine mAb may be needed to clarify the relationship between NK-1 and methamphetamine, we hope that NK-1 will provide useful tools for the detection of methamphetamine and for the study of a clinical basis on therapeutics of narcotist.

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