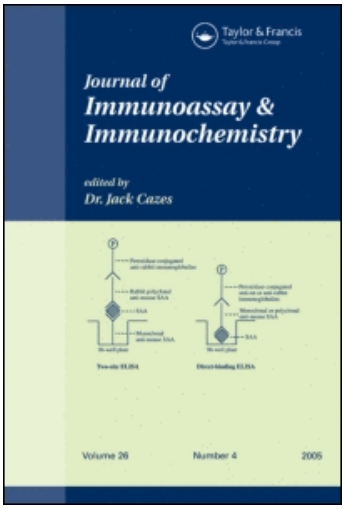

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A Monoclonal Antibody to Hippuric Acid: An Improved Enzyme-Linked Immunosorbent Assay for Biological Monitoring of Toluene Exposure

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Abstract: A novel monoclonal antibody (MAb) to hippuric acid (HA) was prepared using an HA analog, *N*-α-benzoyl-lysine, as an immunogen. An enzyme-linked immunosorbent assay (ELISA) for HA was established using the anti-HA MAb named HA01BL. When the specificity of the MAb was analyzed by the ELISA system, the MAb was revealed to be less reactive to methylhippuric acids and to be more specific to HA than previously reported polyclonal antibodies. The detection limit of HA by the ELISA was approximately 1 μg/mL. The urinary HA concentration determined by the ELISA system correlated well with that obtained by high performance liquid chromatography.

Keywords: Toluene, Monoclonal antibody, Hippuric acid, Biological monitoring, Enzyme-linked immunosorbent assay (ELISA)

INTRODUCTION

Toluene is mainly metabolized to hippuric acid (HA) via oxidation and conjugation, then finally excreted in the urine.[1] Therefore, the urinary HA concentration has been used as an indicator for the biological monitoring of

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occupational toluene exposure.[2] In some countries, such as Germany,[3] the urinary marker of occupational toluene exposure was changed to *o*-cresol. However, HA continues to be employed in many countries, including Japan. In addition, it can still be used as a marker for environmental toluene exposure.

We previously developed an enzyme-linked immunosorbent assay (ELISA) for urinary HA using rabbit anti-HA polyclonal antibody.[4] The ELISA system was sufficiently sensitive and specific, and also had the advantage of being able to assay a large number of samples relatively quickly, compared to commonly used methods such as high-performance liquid chromatography (HPLC).[5–7]

However, employing polyclonal antibodies for ELISA has disadvantages: assay conditions established with one antiserum may not be directly applied to another, and there are problems with reproducibility and reliability of results. This is due to characteristics of polyclonal antibodies, which are not always the same between preparations. For example, the specificities of antisera from two rabbits will be at least slightly different from each other unless the same immunogen was injected.

To overcome this problem, we attempted to prepare a murine monoclonal antibody (MAb) to HA and to establish an ELISA system for HA with the MAb. In the case of an MAb, it is possible to prepare large amounts of homogeneous antibodies with defined specificity and, thus, to continue obtaining highly reproducible ELISA results.

EXPERIMENTAL

Reagents

Reagents for Immunochemical Experiments

N-γ-Maleimidobutyryloxysucciniimide (GMBS) and keyhole limpet hemocyanin (KLH) were purchased from Calbiochem (La Jolla, CA, U.S.A.). *N*-α-Benzoyl-L-lysine (B-Lys) was obtained from Novabiochem (Läufelfingen, Switzerland). *N*-Benzoyl-L-cysteine (B-Cys) was purchased from Peptide Institute, Inc. (Osaka, Japan). HA and methylhippuric acid (MHA) isomers were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). *p*-Aminohippuric acid (p-AHA) and glutaraldehyde were obtained from Wako Pure Chemicals Industry Co., Ltd. (Osaka, Japan). Bovine serum albumin (BSA) and hippuryl lysine were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Horseradish peroxidase conjugated rabbit anti-mouse immunoglobulin antibody and swine anti-rabbit immunoglobulin antibody were from Dakopatts A/S (Glostrup, Denmark). Other reagents used were all analytical grade, unless otherwise indicated.

Reagents for Cell Culture Experiments

RPMI1640 medium was purchased from Nissui Pharmaceuticals Co., Ltd. (Tokyo, Japan). Fetal calf serum (FCS) was obtained from Gibco BRL (Life Technologies, Inc., Grand Island, NY, U.S.A.), inactivated at 56°C for 30 min prior to use and added to RPMI1640 medium to a final concentration of 20% (v/v). Polyethyleneglycol (PEG) 3400 was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), melted by autoclaving (121°C, 30 min) and diluted with FCS-free RPMI1640 medium to make a 50% (w/v) solution. Dimethylsulfoxide was added to the PEG solution to make a final concentration of 13% (v/v). Hypoxanthine-aminopterine-thymidine (HAT) selection medium and hypoxanthine-thymidine (HT) medium were prepared according to the manufacture's instructions with respective supplements purchased from Gibco BRL using RPMI1640 medium containing 20% FCS. Other reagents used were all cell culture grade, unless otherwise indicated.

Preparation and Characterization of Antibodies

Immunogen (B-Lys-KLH Conjugate)

B-Lys-KLH conjugate was prepared as previously described.[4] The ratio of B-Lys to KLH was 57 nmol to 1 mg.

Immunization

On day 0, 200 μg of B-Lys-KLH conjugate was dissolved in 140 μL of PBS and mixed with an equal volume of Complete Freund's Adjuvant (CFA; Difco Laboratories, Inc., Detroit, MI, U.S.A.) to form an emulsion. The emulsion was injected intraperitonially into a female BALB/c mouse (6 wks old, Japan SLC, Inc., Shizuoka, Japan). On days 14 and 28, emulsion prepared with Incomplete Freund's Adjuvant (Difco Laboratories, Inc.) instead of CFA was injected according to the same procedure. The antisera were harvested 2 weeks after the last injection. The titer of the antisera against the immunogen was over 10^6.

Booster injection was carried out subcutaneously with 50 μg of B-Lys-KLH in PBS, 1 month after the last immunization.

Preparation of Hapten Immobilized Plates

B-Lys was immobilized covalently onto Amino-Plates (MS-3696F, Sumitomo Bakelite Co., Ltd., Tokyo, Japan) with 96 wells containing adherent amino groups on their surface, as previously described.[4]

Preparation of Antibody-Producing Hybridomas

Antibody producing hybridoma was prepared as previously described by Oi and Herzenburg[8] with slight modifications. Briefly, splenocytes were obtained from the immunized mouse and mixed with SP2/0 murine myeloma cells at a ratio of 10 : 1. Cell fusion was carried out by adding 0.5 mL of prewarmed PEG solution to the cell pellet, mixing gently with a pipette tip, and consequtively centrifuging at 200 rpm for 2 min, 800 rpm for 2 min and 1000 rpm for 2 min at room temperature. After washing with FCS-free RPMI1640 medium, the fused cells were suspended in RPMI1640 medium containing 20% FCS and cultured in 96-well culture plates at 5×10^5 cells in 100 μL medium per well. All cultures were incubated at 37°C, 5% CO_2 and 100% humidity.

Twenty-four hours after cell fusion, 100 μL of HAT selection medium was added to each well. From 4 to 10 days after cell fusion, half of the medium in all wells was replaced with fresh HAT medium every two days.

The plates were observed under a microscope every day, and supernatants were sampled from wells with well-grown cells and subjected to screening assay.

Screening Assay

The antibody activity in the culture supernatant was examined using hapten-immobilized microplates prepared as mentioned above.

Ten-fold diluted culture supernatant was added to the wells at 50 μL/well and incubated for 1 h at room temperature. After washing with PBS, 50 μL/well of two thousand-fold diluted peroxidase-conjugated anti-mouse immunoglobulin antibody in 1% BSA/PBS was added and incubated for 1 h at room temperature. Bound peroxidase activity was detected by adding 50 μL/well of 10 mg/mL tetramethylbenzidine / 0.06% H_2O_2/0.1 M citrate buffer (pH 5.0) after the plates were washed with PBS. The enzyme reaction was stopped by adding 50 μL/well of 1 M H_3PO_4, then the absorbance at 450 nm was measured on a microplate reader (Model 3550, Bio-Rad Laboratories, Richmond, CA, U.S.A.). Plates without hapten immobilization were used for background.

Cloning

Hybridomas of the wells with anti-B-Lys activity were maintained in HT medium and subjected to cloning by the limiting dilution technique.

ELISA for HA

Preparation of B-Cys-BSA Conjugate

B-Cys-BSA was prepared as previously described.[4] The amount of conjugated B-Cys was estimated to be 12.2 nmol/mg BSA.

ELISA Procedure

Urine samples were diluted ten-fold with 0.5 M sodium phosphate buffer (pH 7.2) prior to the assay in order to minimize interference by ingredients other than HA in urine.

Microplates with 96 wells (Sanko Junyaku Co., Ltd., Tokyo, Japan) were coated with 50 μL/well of 10 μg/mL B-Cys-BSA in PBS for 2 h at room temperature and blocked with 300 μL/well of 1% BSA/PBS for 1 h. Samples were mixed with 0.2 μg/mL monoclonal antibody in 2% BSA/PBS at a ratio of 1:1, then added to the wells at 50 μL/well and incubated at room temperature for 1 h. After washing with PBS, 50 μL/well of two thousand-fold diluted peroxidase-conjugated anti-mouse immunoglobulin antibody in 1% BSA/PBS was added and incubated for 1 h at room temperature. Bound peroxidase activity was detected using the substrate tetramethylbenzidine, as described above.

In the characterization experiments for rabbit polyclonal anti-HA antibody, peroxidase-conjugated anti-rabbit immunoglobulin antibody, 1 mg/mL *o*-phenylenediamine and 2 M H_2SO_4 were used instead of peroxidase-conjugated anti-mouse immunoglobulin antibody, 10 mg/mL tetramethylbenzidine and 1 M H_3PO_4, respectively, and absorbance at 490 nm was measured.

HPLC

Urinary HA and MHA isomers were analyzed by HPLC according to Sakai et al.[7] with some modifications as described previously.[4]

RESULTS

Characteristics of the MAb

Through screening and cloning, a clone producing anti-B-Lys antibody was established and named HA01BL. The immunoglobulin subclass of HA01BL antibody was determined with Mouse Typer Sub-Isotyping Panel (Bio-Rad Laboratories, Hercules, CA, U.S.A.) according to the manufacturer's instructions, and was proved to be mouse IgG1 which was composed of γ1 heavy chain and κ light chain.

Establishment of ELISA for HA with HA01BL

The ELISA procedure was essentially the same as that with rabbit polyclonal anti-HA antibodies.

A schematic diagram of the established ELISA procedure is shown in Fig. 1. A typical standard curve of the ELISA system is shown in Fig. 2, along with the reaction curve obtained with the various cross-reacting compounds described below. The detection limit was around 1 μg/mL and the effective assay range was from 1 to 500 μg/mL.

Specificity of HA01BL Antibody

The cross-reactivity of HA01BL MAb with certain HA-related compounds was analyzed by subjecting these compounds to the ELISA (Fig. 2). The

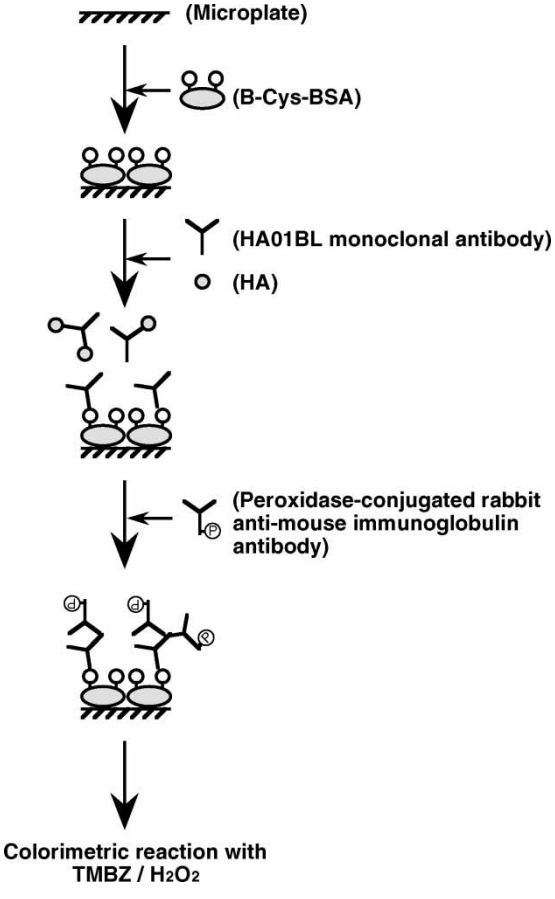

Figure 1. Schematic presentation of the HA ELISA.

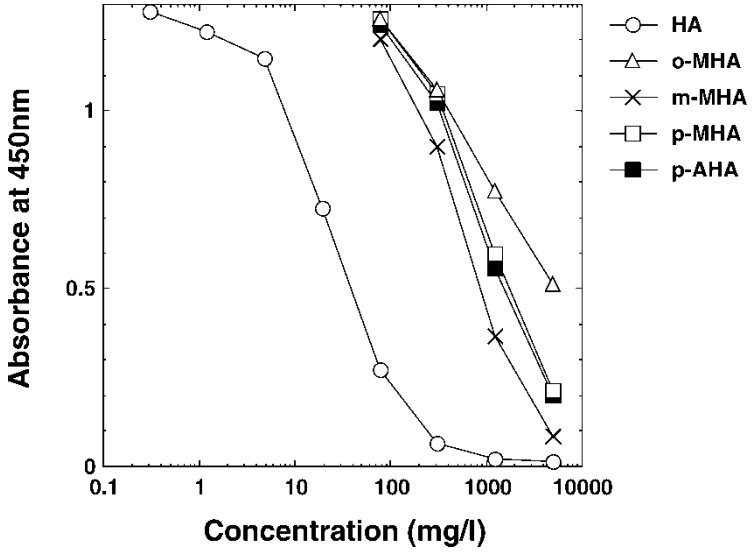

Figure 2. The reactivity of HA01BL monoclonal antibody to hippuric acid (HA), methylhippuric acid (MHA) isomers and *p*-aminohippuric acid (p-AHA). The reactivity to HA shows the standard curve of the ELISA for HA.

results with previously reported rabbit polyclonal anti-HA antibody are shown in Fig. 3. Cross-reactivities calculated from half-maximum response values (EC50) including those for compounds which are not shown in Figs. 2 and 3 are summarized in Table 1.

Determination of HA Concentration in Urine by ELISA

Urine samples obtained from industrial workers exposed to toluene were analyzed by the ELISA. Figure 4 shows the correlation between the urinary HA concentration determined by the ELISA and by HPLC ($r^2 = 0.952$).

DISCUSSION

We have previously reported preparation of rabbit polyclonal antibody reactive to HA.[4] The polyclonal antibody was sufficiently specific to HA for determination of human urinary HA, and the resultant ELISA system was shown to be a potential alternative to commonly used HPLC.

However, there are some crucial problems in employing the polyclonal antibody in the ELISA system in biological monitoring which requires high reproducibility and reliability. The most important problem is a difficulty in obtaining a large amount of antibodies with identical reactivity. Usually,

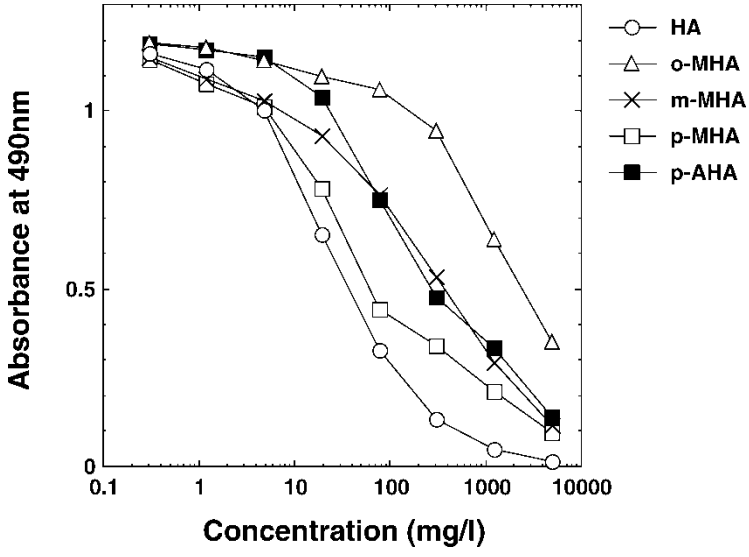

Figure 3. The reactivity of rabbit anti-HA polyclonal antibody to HA, MHA isomers and p-AHA.

antisera obtained from several rabbits are not the same among rabbits, even if the same immunogen is injected with the same schedule. Therefore, assay conditions established with an antiserum from one rabbit may not be directly applied to that from another rabbit. Another problem lay in the specificity of the antibodies. The polyclonal antibody reported in the previous paper

Table 1. Cross-reactivites of HA01BL monoclonal antibody and rabbit anti-HA polyclonal antibody

	HA01BL		Rabbit anti-HA polyclonal antibody	
Compound	EC50 (mg/L)	Cross-reactivity (%)	EC50 (mg/L)	Cross-reactivity (%)
HA	25.3	—	24.3	—
o-MHA	2507.7	1.01	1463.8	1.66
m-MHA	613.4	4.12	209.0	11.60
p-MHA	1095.4	2.31	39.9	60.98
p-AHA	967.4	2.61	163.0	14.88
Benzoic acid	1800.5	1.40	4667.3	0.52
Mandelic acid	1525.6	1.66	ND	ND
Hippuryl lysine	1051.0	2.41	1240.3	1.96

ND; No reactivity was detected up to 5000 mg/L.

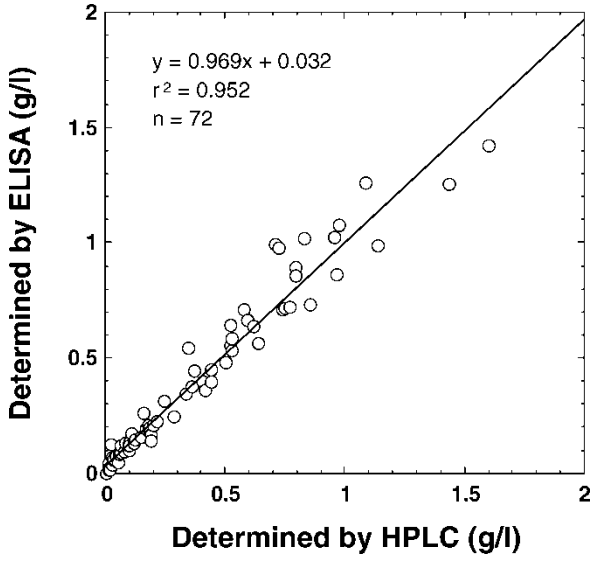

Figure 4. Comparison of the HA concentrations (g/L) obtained by HPLC and ELISA.

showed significant cross-reactivity to other compounds such as MHA isomers, although it did not affect the results of urinary HA analysis. These problems were considered to reduce the reliability of the results of the ELISA.

In order to overcome these problems, a murine MAb was established, because, a large amount of monospecific and homogeneous antibody can be prepared in the case of MAb by simply expanding the culture scale. B-Lys-KLH was used as the immunogen because polyclonal anti-HA antibody was successfully prepared in a previous report.[4]

From hybridomas prepared from one mouse, a clone which produces IgG1 anti-HA MAb was established. The hybridoma, named HA01BL, grew well and stably produced antibody to its culture medium. Therefore, the problem of supply of the antibody was solved.

The ELISA procedure was established using HA01BL MAb (Fig. 1). As shown in Fig. 2, the effective assay range was about 1 to 500 mg/L, which is almost the same as for polyclonal antibody-based ELISA (Fig. 3). In addition, the EC50 values to HA of HA01BL and the polyclonal antibody were nearly the same (Table 1), therefore the sensitivity of the ELISA for HA using HA01BL MAb would be comparable to that using the polyclonal antibody which was sensitive enough to estimate urinary HA as described before.[4]

The specificity of the resultant MAb was analyzed and compared with that of rabbit polyclonal antibody using MHA isomers (Figs. 2 and 3). As noted, the MAb (HA01BL) is more specific to HA than the polyclonal antibody. The difference was most apparent when the reactivities to HA and p-MHA were

compared. In the case of HA01BL MAb, more than thirty times the amount of p-MHA was needed to obtain a similar reaction of HA while 1.5-fold p-MHA was enough for polyclonal antibodies. The cross-reactivity with several compounds structurally related to HA was assessed by comparing the half-maximum response value (EC50) of the ELISA (Table 1). The HA01BL MAb showed less than 5% cross-reactivity to all compounds tested. This indicated that the MAb strictly recognized the absence of any additional substitutions on the benzene ring of HA, because MHA isomers and p-AHA were less reactive. In contrast, m-MHA, p-MHA and p-AHA were reactive to the polyclonal antibody at more than 10%. The MAb was also considered to recognize the -COOH group of the glycine moiety that remained free, because hippuryl lysine, in which lysine was bound to the group, showed little reactivity. From these results, it can be concluded that HA01BL MAb has apparent advantages over the polyclonal antibody in ELISA for HA in specificity and reliability.

The cross-reactivity of the polyclonal antibody shown in Table 1 is different from that in our previous paper.[4] This was due to the difference of the assessment method. In the previous paper, the apparent concentration values obtained with 500 mg/L of cross-reacting compounds were compared and the cross-reactivities were underestimated compared to those in the present report.

Finally, human urine samples obtained from industrial workers exposed to toluene were measured by the MAb-based ELISA and conventional HPLC. The correlation between the HA concentration determined by the ELISA and by HPLC was satisfactorily high ($r^2 = 0.952$) (Fig. 4). This result strongly supports that the ELISA can be a practical alternative to HPLC in the biological monitoring of toluene exposure.

As mentioned in our previous paper, the ELISA system has an advantage over HPLC in requiring a shorter time to process a large number of samples. The newly established anti-HA MAb assures a continuous supply of homogeneous antibody and reliability of the ELISA, thus increasing the opportunity of the ELISA to be employed.

To our knowledge, this is the first report on a MAb against organic solvent metabolites. Application of the antibody to sophisticated immunological detection systems such as multilayer film immunoassay[9] and liposome immune lysis assay[10] should make human urinary HA determination simpler, easier, and faster. The newly established anti-HA MAb assures the specificity of the antibody, thus promoting the development of new determination systems for human urinary HA.

CONCLUSION

An ELISA for HA was established using the anti-HA MAb named HA01BL. The MAb was considered to be more advantageous than polyclonal antibodies in its specificity and stability, because unlimited amounts of homogeneous

antibody with defined specificity could be obtained. The ELISA system was shown to be a potential alternative to commonly used HPLC for the biological monitoring of toluene exposure.

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