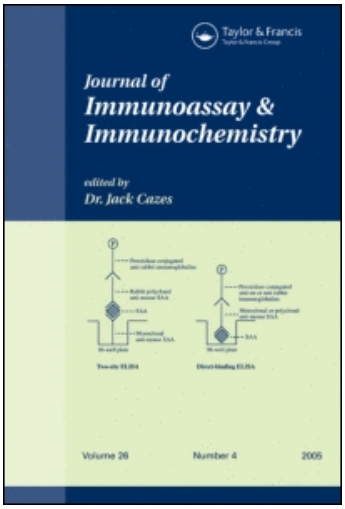

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DEVELOPMENT AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES REACTIVE WITH PARAQUAT

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ABSTRACT

A set of three anti-paraquat monoclonal antibodies(MoAbs), named APM-1, APM-2 and APM-3, has been isolated. In order to evaluate the ability of these MoAbs to recognize various kinds of bipyridyl herbicides and similar congeners of paraquat, a competition enzyme-linked immunosorbent assay (ELISA) using avidin-biotin complex (ABC) was developed. All three antibodies strongly recognized paraquat and slightly did the other analogs. These three MoAbs are therefore advantageous to a toxicological study of paraquat and of its localization in tissues. (KEY WORDS: Paraquat, Monoclonal antibodies, ELISA, Avidin-biotin complex (ABC))

INTRODUCTION

Paraquat (1,1'-dimethyl-4,4'-bipyridinium) is widely used as a herbicide. While there is no evidence for deleterious side effects in normal use, accidental or deliberate ingestion of paraquat can be lethal (1,2). In the field of forensic toxicology,

establishment of the method for the identification and quantification of paraquat in samples is required. The analytical procedure should be simple enough for a relatively untrained researcher to perform rapidly.

Immunoassays cover these criteria and have proven to be effective in detecting clinically important compounds. In order to establish a highly sensitive immunoassay system for paraquat , antibodies which strongly recognize paraquat are needed. Niewola et al. (3) had described production of a MoAb against paraquat, but this antibody shows twice as much affinity for diethyl paraquat as for paraquat, therefore the specificity to the latter was not satisfactory .

We report here the development and characterization of three specific MoAbs against paraquat (named APM-1, APM-2 and APM-3).

MATERIALS AND METHODS

Chemicals

Paraquat dichloride (1,1'-dimethyl-4,4'-bipyridinium dichloride) and 6-bromohexanoic acid were purchased from Aldrich Co., U.S.A. Monoquat chloride

(1-methyl-4,4'-bipyridinium chloride), diethyl paraquat diiodide (1,1'-diethyl-4,4'-bipyridinium diiodide), diquat dibromide (1,1'-ethylene-2,2'-bipyridinium dibromide) and morfamquat dichloride (1,1'-bis (3,5-dimethyl-morpholino-carboxymethyl) 4,4'-bipyridinium dichloride) were generous gifts from Plant Protection Division, ICI, PLC. MPTP chloride (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine chloride) and MPP chloride (1-methyl-4-phenyl-pyridine chloride) were purchased from Research Biomedicals Inc. , U.S.A. [^{14}C]Methyl iodide (specific activity, 58 mCi/mmol) and [methyl[^{3}H]]paraquat dichloride (specific activity, 2.5 Ci/mmol) were obtained from Amersham International plc. Flat-bottomed polystyrene microtiter plates (Immuno Plate I) were obtained from Nunc, Denmark. Biotinylated horse anti-mouse IgG(H+L) and a Standard Vectastain ABC (biotinylated alkaline phosphatase) Kit were purchased from Vector Laboratories, U.S.A. Anti-mouse IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgM and IgA antisera were obtained from Serotec , Co. Ltd., U.K. and anti-mouse IgG antiserum from Organon Teknika Corp. - Cappel Products , U.S.A. All other reagents were obtained from Nakarai Chemical, Ltd., Japan.

Preparation of Antigen

Labelled 1-methyl-4,4'-bipyridinium iodide was prepared by the method of Fatori and Hunter with a

slight modification (4). Ten g of 4,4'-bipyridyl and 9.1 g of methyl iodide containing [^{14}C]methyl iodide (0.1 mCi) were reacted in 100 ml of dry chloroform, and stirred overnight at room temperature. After stirring, 1-methyl-4,4'-bipyridinium iodide was collected by filtration, and washed with dry chloroform and then stored in a vacuum desicator over silica gel.

1-Methyl,1'-hexanoic acid-4,4'-bipyridinium (MHBP) as a paraquat hapten was synthesized according to the method of Niewola et al. (5) with a slight modification and coupled to bovine serum albumin (BSA) and gelatin with carbodiimide (FIGURE 1). The purity of the MHBP was analysed by mass spectrometry and thin layer chromatography (TLC) on plates coated with cellulose. Mass spectrometry was performed on JEOL, JNS-DX300. The ionizing voltage was adjusted to 70 eV. The degree of conjugation with carrier proteins was calculated to be about 20 and 12 mol of the hapten per BSA (mol.wt. 68,000) and gelatin (average mol.wt. ca. 100,000), respectively, on the basis of the radioactivity.

In order to develop a competition ELISA, a diazo-coupled paraquat-BSA conjugate was synthesized. One hundred and seventeen mg of 4-amino-D,L-phenylalanine and 12 mg of sodium bromide were dissolved in 6 ml of water and acidified with 120 µl of conc. HCl

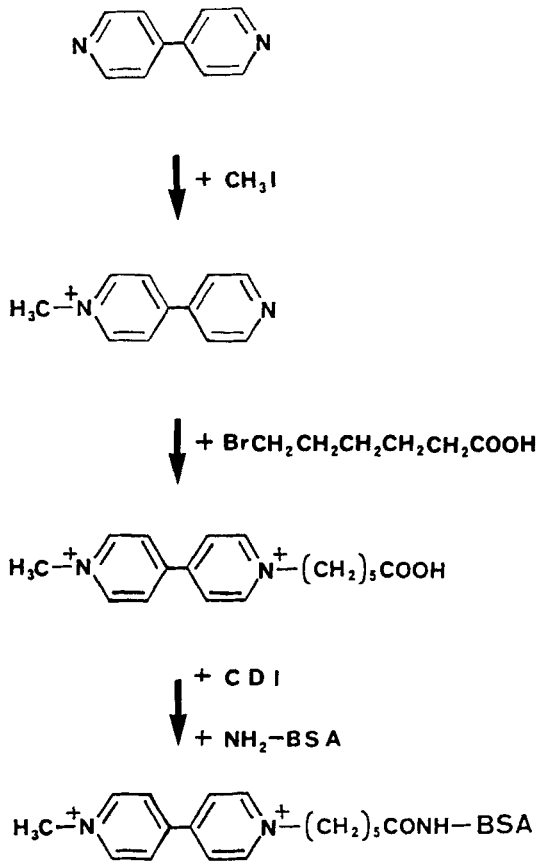


FIGURE 1. Reaction scheme for preparation of immunogen.

solution. Then 1 ml of water containing 55 mg of $NaNO_2$ was added dropwise to the mixture and the solution was mixed for 10 min on an ice bath. The excess $NaNO_2$ was then neutralized with 1 % ammonium sulfamate aqueous solution. Thirty three mg of paraquat dichloride containing about 500,000 dpm [^{3}H]paraquat were dissolved

in 5 ml of 0.1M borate buffer (pH 9.0) and 1.424 ml of the diazotized 4-amino-D,L-phenylalanine solution, which is equivalent to the concentration of paraquat, were added to the paraquat solution. The mixture was stirred for 6 h on the ice bath maintaining the pH between 8.0 and 8.5. Thirty mg of BSA in 1 ml of water and 38 mg of carbodiimide in 0.5 ml of water were added to the diazo-coupled paraquat derivative solution and stirred for 24 h at room temperature maintaining the pH between 8.0 and 8.5. Further 19 mg of carbodiimide were added to the reaction mixture and kept stirring for 24 h. Finally the mixture was dialyzed against running water for 48 h. The resulting conjugates contained 25 mol of the diazo-coupled paraquat derivative /mol of BSA.

Monoclonal Antibody Production

Six-week-old BALB/C mice were first immunized intraperitoneally (ip) with 250 μg of paraquat-BSA in saline emulsified 1:1 in Freund's complete adjuvant and then received the intraperitoneal injection of 250 μg of paraquat-BSA only in saline monthly for 6 months. Three days after the final injection, spleens were removed, fused with P3U1 myeloma cell line using PEG 1500

and then were grown under the conditions as described by Köhler and Milstein (6).

Hybridomas producing antibody were screened by an ELISA. In this procudure, 96-well microtiter plates were coated with 1.0 μg/well of paraquat-gelatin or BSA alone in 0.01M carbonate buffer (pH 9.6) over night at 4°C, washed three times with 0.01M phosphate-buffered saline (PBS) containing 0.05 % sodium azide (pH 7.4), filled with PBS containing 1 % gelatin for blocking and left for 1 h at 37°C. After removal of the blocking solution, each hybridoma supernatant was applied, and the following procedure of the screening test was described below Step 2 of a competition ELISA procedure.

In this screening procedure, the hybridoma wells which are not only positive against paraquat-gelatin used as a solid phase in the ELISA but also negative against BSA were selected as the hybridomas producing antibody. These hybridoma cells from positive wells were subcloned twice by a limiting dilution to ensure their monoclonal origins. Isotype analyses were performed by the double-diffusion technique of Ouchterlony (7).

ELISA

A competition ELISA was developed to quantify the amount of free antigen in solution and to evaluate

the cross-reactivity of various bipyridyl herbicides and other analogs structurally similar to paraquat with the antibodies. The principal ELISA procedure was carried out as described previously (8).

Step 1: Wells of a flat-bottomed microtiter plate were first coated with 1.0 µg diazo-coupled paraquat-BSA in 100 µl of 0.01M carbonate buffer(pH 9.6) over night at 4°C, washed three times with PBS and filled with PBS containing 1 % gelatin for blocking and left for 1 h at 37°C.

Step 2: After removal of blocking solution, 50 µl of diluted MoAb with PBS containing 0.1 % gelatin and 0.05 % Tween 20 (PBSG) and 50 µl of PBSG containing known amounts of paraquat or its analogs were added in duplicate to individual wells and incubated for 2 h at 37°C.

Step 3: After washing three times with PBSG, 100 µl of biotinylated horse anti-mouse IgG (H+L) (6 µg protein/ml in PBSG) were added to the wells.

Step 4: Following incubation for 60 min at 37°C, the plate was washed three times with 0.01M Tris-buffered saline (pH 7.4 TBS) and 100 µl of ABC solution, which was prepared by the addition (5 µl) of each of A and B reagents of the Vectastain ABC (biotinylated alkaline phosphatase) Kit to 1 ml of TBS, were added to the wells.

Step 5: Following incubation for 30 min, the plate was again washed with TBS and 100 µl of a chromogenic

solution containing 100 mg of phenylphosphoric acid disodium salt and 116 mg of 4-aminoantipyrine in 105 ml of 0.05M carbonate buffer (pH 10.0) were added to the well (9). After incubation for 30 min, the enzymic reaction of alkaline phosphatase was stopped by the addition of 100 µl of 1.2 % potassium ferricyanide aqueous solution. The absorbance in each well was determined at 492 nm on a two-wave length microplate photometer (Corona MTP-22, Japan).

RESULTS

Purity of MHBP

The purity of MHBP (M.W. 286, as a free form) was confirmed by mass spectrometry using the direct injection method (DI-MS method) as shown in FIGURE 2. Three major peaks were observed. They are 285 m/z (M - H), the ion peak of the aggregated molecule; and 177 and 156 were 1- methyl - 4, 4'- bipyridinium and 4, 4' -bipyridyl, respectively. TLC in a 12:3:5 (v/v) n-butyl alcohol: acetic acid: water system showed a single spot of MHBP (Rf, 0.25).

Hybridoma Production

After the cell fusion, hybridomas in wells of more than 90 % of the microculture plates were

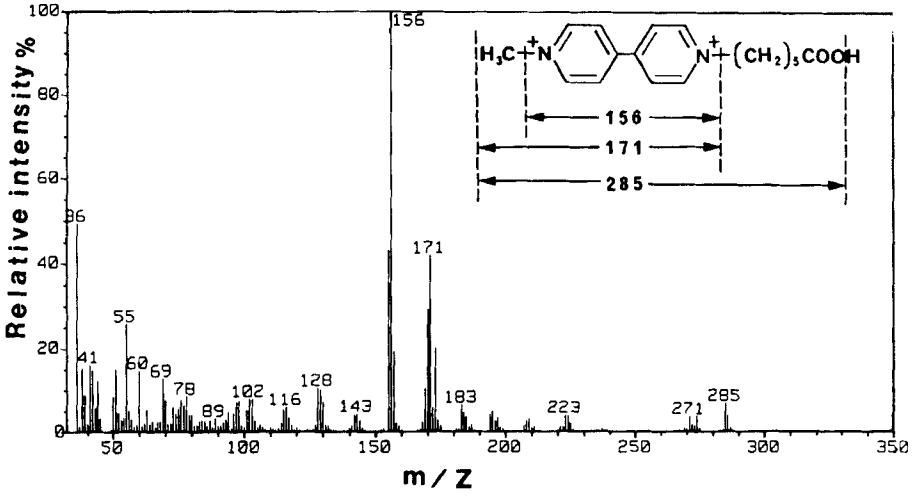


FIGURE 2. Mass spectrum of MHBP; Ionization energy, 70 eV.

observed. Antibodies produced in hybridoma supernatants were first screened by the ELISA coated with paraquat-gelatin as the solid phase, and then the antibodies non-reactive with BSA among the antibody-positive clones were selected by use of the ELISA coated with BSA alone. Finally three clones were established, and each MoAb of the clones recognized the hapten but did not bind to the carrier protein. The subclass of mouse immunoglobulins in these clones (APM-1, APM-2 and APM-3) was determined to be IgG_1, IgG and IgG, respectively.

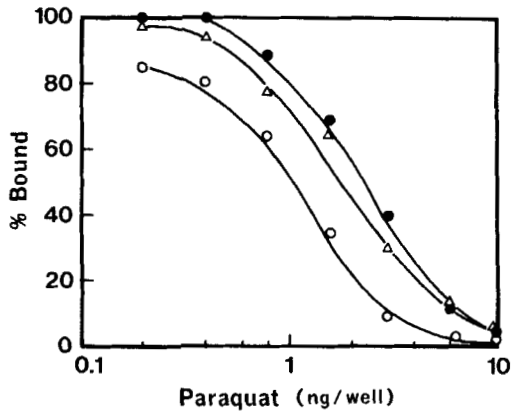


FIGURE 3. Inhibition of binding of MoAbs to diazo-coupled paraquat-BSA in the ELISA by paraquat dichloride: closed circle; APM-1, open circle; APM-2 and open triangle; APM-3.

ELISA

When the diazo-coupled paraquat-BSA as the solid phase was used for the competition ELISA system, it was possible to work with 1:3200, 1:200 and 1:800 dilutions of each culture fluid (APM-1, APM-2 and APM-3), respectively. FIGURE 3 shows the behavior of three different MoAbs for paraquat ELISA. The absorbance was decreased according to increasing amounts of paraquat dichloride in the reaction mixture and , the decrease in the color development was almost linear in the range of 1 ng to 10 ng of paraquat. In this ELISA system, the amounts of paraquat dichloride causing

a 50 %inhibition with three MoAbs of APM-1, APM-2 and APM-3 were 2.5, 1.1 and 2.1 ng, respectively (FIGURE 3).

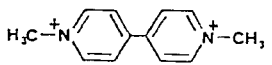
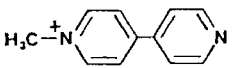
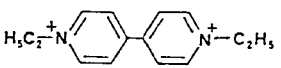
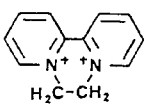
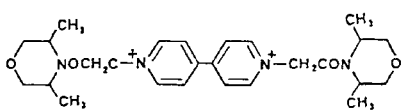
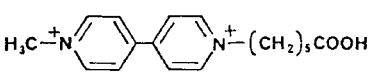
Characterization of MoAbs

The specificity of these MoAbs was evaluated by cross-reactivity studies with paraquat, bipyridyl derivatives, MPTP and MPP (TABLES 1 and 2). MHBP as the paraquat hapten-spacer and diethyl paraquat showed relatively significant cross-reactivities with these three MoAbs. Morfamquat could not bind to all MoAbs but the only APM-2 showed slight binding affinity for MPTP. The other congeners were poorly recognized by all MoAbs (TABLES 1 and 2). However, all these MoAbs that were produced from the different clones strongly and almost similarly recognized paraquat , but little did the part of spacer of the paraquat hapten (MHBP-BSA).

DISCUSSION

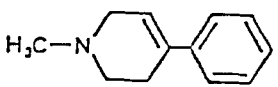
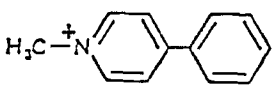
TABLE 1 shows that diethyl paraquat and monoquat are slightly recognized by these MoAbs, the affinity for the former being relatively stronger than that for the

TABLE 1
CROSS-REACTIVITY of THREE DIFFERENT MoAbs with BIPYRIDYL DERIVATIVES.

Reagent	Structure	Cross-reactivity(%) APM-1	APM-2	APM-3
Paraquat		100	100	100
Monoquat		4.3	2.0	1.0
Diethyl paraquat		22.0	8.1	11.8
Diquat		4.4	0.3	1.4
Morfamquat		0.004>	0.002>	0.004>
MHBP[a]		36.9	20.1	19.9

[a]1-Methyl,1'-hexanoic acid-4,4'-bipyridinium

TABLE 2
CROSS-REACTIVITY of THREE DIFFERENT MoAbs with SIMILAR ANALOGS to PARAQUAT.

Reagent	Structure	Cross-reactivity(%) APM-1	APM-2	APM-3
MPTP		0.08	0.0009>	0.03
MPP^+		0.5	0.9	0.4

latter. This finding suggests that the cross-reactivity with these analogs depends on the structural similarity to the hapten (MHBP). Morfamquat, which has the 4,4'-bipyridyl ring masked by bulky groups, was not detected by three MoAbs at all, however, diquat was slightly recognized although MPP and MPTP were little detected (TABLES 1 and 2). These results suggest that both a bipyridyl ring and a methyl group of either 1- or 1'-position of paraquat are contributing to the epitope.

Some variations in antibody-binding selectivity were expected to be of the different clonal origins of hybridomas. APM-1 was the least specific of these MoAbs and showed the relatively strong binding affinity for both MHBP and diethyl paraquat (TABLE 1), suggesting that the epitope recognized by APM-1 contains some parts of spacer of the antigen. APM-3 showed almost similar affinities for congeners of paraquat to APM-1, however, the epitope of APM-3 seems to be limited in the relatively narrower part of the spacer than that of APM-1, because of the fact that the former MoAb showed lower binding affinity for MHBP and diethyl paraquat than the latter one (TABLE 1). On the other hand, since APM-2 scarcely recognized diquat, MPTP and MPP, the major part of the epitope of APM-2 appears to be the bipyridyl ring of paraquat (TABLES 1 and 2).

The anti-paraquat MoAb reported by Niewola et al. (3) showed over twice as much affinity for diethyl

paraquat as for paraquat and the general specificity of the MoAb was rather inferior to that of polyclonal antibody they demonstrated, whereas our MoAb (APM-2) showed 8 % cross-reactivity with diethyl paraquat. Moreover, the binding affinity of our MoAb for monoquat was much lower than that of their one (3) (TABLE 1). Judging from these results, we will be able to evaluate that our MoAb, APM-2, is significantly more specific to paraquat than Niewolas' one. However, the sensitivity of Niewolas' MoAb was shown to be much superior to that of ours. This may be attributed to differences in thebinding mode of the coating antigen in the ELISA system, its carrier and/or in particular specificity of MoAb. The competition ELISA using of three MoAbs demonstrated here was capable of detecting nanogram amounts (1 to 10 ng) of paraquat. In toxicological and clinical practice, this sensitivity in the present ELISA, however, is thought to be enough usable, especially in comparison with instrumental analyses of paraquat such as gas chromatography (10) and high pressure liquid chromatography (11).

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