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Monoclonal anti-diuron antibodies prevent inhibition of photosynthesis by diuron

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Abstract **Two monoclonal anti-diuron antibodies were generated that bind to diuron with an extremely low equilibrium dissociation constant. The antibodies prevented and restored in vitro and in vivo the diuron-dependent inhibition of photosynthesis. In isolated thylakoids prepared from spinach leaves (*Spinacia oleracea* L.) the diuron-inhibited Hill reaction was reconstituted immediately after the addition of the monoclonal antibodies. The antibodies also restored the diuron-dependent inhibition of the photosynthetic oxygen evolution of the cell wall-deficient mutant *cw15* of the green alga *Chlamydomonas reinhardtii* Dangeard.** **© 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Diuron; Anti-diuron antibody; Photosynthesis; Hill reaction; *Chlamydomonas reinhardtii cw15*

1. Introduction

The herbicide diuron (also known as DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea) is a potent inhibitor of photosynthesis and has been used in agriculture for more than 50 years to inhibit the growth of weeds [1]. It reversibly binds to the exchangeable quinone (Q_B) site of the D1 protein of photosystem II and thus effectively blocks the electron flow from the Q_A site [1,2]. Diuron, therefore, inhibits both the photosynthetic oxygen evolution and the reduction of the terminal electron acceptor, $NADP^+$, which finally turns off the reductive pentose phosphate cycle. In addition to that, diuron has also numerous detrimental effects on non-photosynthetic prokaryotic and eukaryotic organisms [3,4]. Sensitive methods are therefore needed to identify and quantify the herbicide in soil, water and biological materials. Immunoassays based on monoclonal anti-diuron antibodies are regarded as especially useful for such purposes [5].

In the course of our efforts to build up such an immunoassay two newly generated anti-diuron antibodies with an extremely low equilibrium dissociation constant were also checked for their ability to protect plants from the photosynthesis-inhibiting effect of diuron. In an in vitro approach thylakoids isolated from spinach (*Spinacia oleracea* L.) leaves were used to measure the light-dependent reduction of DCPIP (2,6-dichlorophenol indophenol). In another series of experiments the rate of whole cell photosynthesis was monitored polarographically using the cell wall-deficient mutant *cw15* of the unicellular green alga *Chlamydomonas reinhardtii* Dangeard [6]. In both cases, the inhibition by diuron could be reversed by the addition of the monoclonal anti-diuron antibodies.

2. Materials and methods

2.1. Chemicals, antigens and immunogens

The structures of diuron and the diuron-related substances used in these experiments are shown in Fig. 1.

The herbicides diuron, monolinuron and methabenzthiazuron were purchased from Riedel-de-Haën (Seezle, Germany). The diuron derivative POCc9 (3-[3-(3,4-dichlorophenyl)-1-methyl-ureido]-propionic acid) was synthesized by reacting 1,2-dichloro-4-isocyanatobenzene with 3-methylamino-propionitril to form 1-(2-cyano-ethyl)-3-(3,4-dichlorophenyl)-1-methylurea and converting this product to POCc9 by hydrolysis in NaOH.

For immunization and screening for monoclonal antibodies the diuron derivative POCc9 was coupled to KLH (keyhole limpet hemocyanin) and BSA (bovine serum albumin) by means of 1-ethyl-3-(dimethylaminopropyl)-carbodiimide [7]. The hapten to protein ratio of the BSA conjugates was determined by MALDI-TOF MS (matrix-assisted laser desorption/ionization time of flight mass spectrometry) as 9–10 POCc9 residues per BSA molecule.

2.2. Immunization and hybridoma technology

C57Bl/6 mice were immunized twice with the KLH–POCc9 conjugate. For hybridoma production spleen cells were fused with myeloma X63-Ag8.653 cells using polyethylene glycol 1550 (Sigma-Aldrich, Taufkirchen, Germany) according to a modification of the method of Köhler and Milstein [8]. Selected hybrids were cultivated, cloned, subcloned and stored frozen in liquid nitrogen according to standard methods [9]. Antibodies were purified from culture media by protein A affinity chromatography [10].

2.3. Immunoassays

Culture media were screened for antibodies in an enzyme immunoassay according to the following incubation sequence: BSA–POCc9 conjugate adsorbed to the solid phase, phosphate-buffered saline (PBS) containing 5% (v/v) neonatal calf serum to block free protein binding sites, monoclonal anti-diuron antibodies, horseradish peroxidase-labeled anti-mouse Ig antibodies (Sigma-Aldrich, Taufkirchen, Germany), and *o*-phenylenediamine and H_2O_2 as substrate. The enzyme reaction was terminated by 2 M H_2SO_4, supplemented with 50 mM Na_2SO_3, and the absorbance was monitored at 492 nm.

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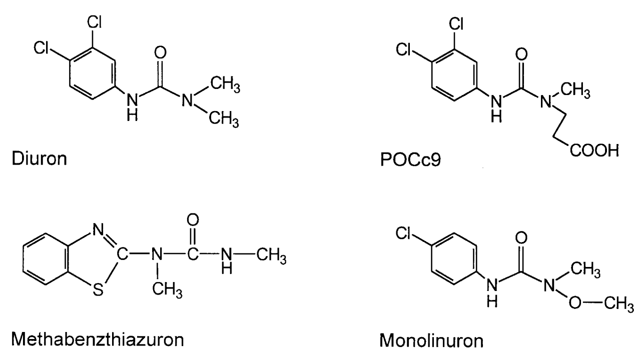

Fig. 1. Structures of diuron and the diuron-related substances used in the present experiments.

The cross-reactivity of the anti-diuron antibodies with the two other herbicides used in these experiments was measured in a competitive enzyme immunoassay. For this purpose purified antibodies, diluted from 1.3 to 15 nM (0.2–2.3 μg/ml), were preincubated with varying concentrations of the herbicide (8 pM to 2 mM dissolved in PBS containing 1% (v/v) dimethylformamide) and subsequently tested for binding to immobilized BSA–POCc9.

For each herbicide the IC_{50} was determined that results in a 50% reduction of the signal and the cross-reactivity of the antibodies was calculated according to the following equation:

$$\text{cross-reactivity}\ (\%) = IC_{50\ \text{diuron}}/IC_{50\ \text{test substance}} \times 100$$

(The $IC_{50\ \text{diuron}}$ is the value obtained for diuron and the $IC_{50\ \text{test substance}}$ is the value for the tested herbicide.)

2.4. Subclass determination

The subclass of the anti-diuron antibodies was determined by an enzyme immunoassay using the following incubation sequence: BSA–POCc9 adsorbed to the solid phase (10 μg/ml, 80 μl/well), monoclonal anti-diuron antibodies, biotinylated subclass-specific goat anti-mouse Ig antibodies (Southern Biotechnology Associates, Inc., Birmingham, AL, USA), alkaline phosphatase-conjugated streptavidin (Boehringer, Mannheim, Germany), and 4-nitrophenylphosphate as substrate. The reaction was terminated by 1 N NaOH and the absorbance was measured at 405 nm.

2.5. Determination of the equilibrium dissociation constant

The equilibrium dissociation constant of the antibodies was determined using a BIACORE® 2000 (Amersham Pharmacia, Uppsala, Sweden) as described previously [11].

2.6. Hill reaction

Thylakoid membranes were isolated from spinach leaves by a modified version of the procedure described by Yocum [12]. Spinach (*S. oleracea* L. cv. *Matador*) plants were grown in soil under controlled conditions (10 h light, approximately 200 μmol quanta m^{-2} s^{-1}, 17°C; 14 h dark, 14°C). Mature leaves (approximately 3 g fresh weight) were homogenized in 25 ml of cold phosphate–sucrose buffer (50 mM KH_2PO_4/K_2HPO_4 pH 8.0, 10 mM NaCl, 5 mM $MgCl_2$, and 0.4 M sucrose). The homogenate was filtered through Miracloth and the resulting filtrate was centrifuged for 5 min at $2000\times g$. The pelleted chloroplasts were resuspended in phosphate–sucrose buffer to give a chlorophyll concentration of 50–60 μg/ml and were stored in the dark on ice prior to use. The reaction mixture contained, in a final volume of 2 ml, 50 mM potassium phosphate (pH 7.0), 10 mM NaCl, 0.052 mM DCPIP, and approximately 6 μg/ml chlorophyll. Diuron and proteins were added as indicated. The diuron stock solution was in ethanol. The final concentration of ethanol was less then 1% (v/v). At regular intervals DCPIP reduction was monitored photometrically at 578 nm [13]. The chlorophyll content of the chloroplast preparation was quantified according to Arnon [14].

2.7. Photosynthesis of algal cells

The cell wall-deficient mutant *cw15* of *C. reinhardtii* Dangeard [6] was grown in modified high salt minimal medium supplemented with Proteose-Pepton No. 3 and yeast extract (0.2% (w/v) each, Difco, Becton Dickinson, Sparks, USA) in a continuous light–dark regime (12 h light; 150 μmol quanta white light m^{-2} s^{-1}; 12 h darkness; 30°C). The cell suspension was aerated with air containing 2% (v/v) CO_2. Prior to use the cells were pelleted by centrifugation (10 min at $800\times g$; 4°C) and resuspended in 50 mM potassium phosphate buffer (pH 7.5) to give an optical density of 0.6–0.8 at 800 nm.

The photosynthetic oxygen production of the cells was monitored using an oxygen electrode. Aliquots (800 μl each) of the cell suspension were transferred to the reaction vessels and 20 μl 250 mM $NaHCO_3$ was added. Diuron and/or proteins were added as indicated. The final volume of the reaction mixture was 1 ml containing approximately 6 μg chlorophyll. Cells were adapted in the dark (30°C) and were then illuminated using white light (1000 μmol quanta m^{-2} s^{-1}).

The chlorophyll content of the algal cells was determined according to Böger [15].

3. Results and discussion

3.1. Characterization of monoclonal anti-diuron antibodies

Several hybridomas were obtained that produced antibodies binding both immobilized BSA–POCc9 and soluble diuron. Two monoclonal antibodies, designated as B91-KF5 and B91-CG5, were used for the present experiments. Both antibodies were of immunoglobulin subclass IgG1.

Antibody B91-KF5 did not cross-react with methabenzthiazuron and cross-reacted only weakly with monolinuron (cross-reactivity 0.2%). Antibody B91-CG5 did not cross-react with methabenzthiazuron and monolinuron. Both antibodies showed no cross-reactivity with decyl plastoquinone, the physiological ligand of the D1 protein.

Both antibodies exhibited the same high affinity for diuron. The K_D value was 2.2×10^{-11} M for B91-CG5 and 2.3×10^{-11} M for B91-KF5. The binding constant k_{on} was 6×10^5 s^{-1} M^{-1} for B91-KF5 and 2.3×10^5 s^{-1} M^{-1} for B91-CG5.

3.2. Prevention of diuron-dependent inhibition of the Hill reaction by the anti-diuron antibodies

The Hill reaction was monitored under the influence of diuron with and without antibodies using thylakoids isolated from spinach leaves and DCPIP as electron acceptor. A diuron concentration of 0.5 μM resulted in almost complete inhibition of the DCPIP reduction. When diuron was preincubated with the B91-KF5 antibody and this mixture was then

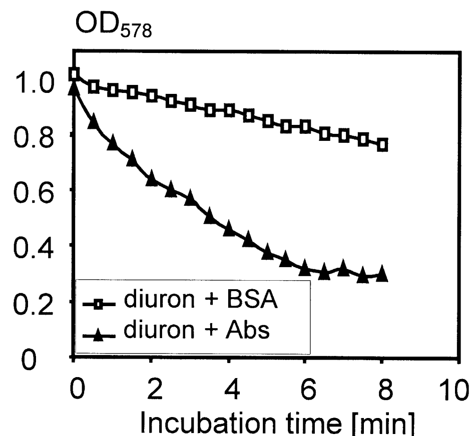

Fig. 2. Inhibition of the light-dependent DCPIP reduction by diuron and neutralization of this inhibition by anti-diuron antibodies. Diuron (0.5 μM) and anti-antibody B91-KF5 (Abs; 0.75 μM) or diuron and BSA (0.75 μM) as control were preincubated for 1 h at room temperature. Subsequently, an aliquot of the mixtures was transferred to the Hill reaction mixture and the Hill reaction was measured for 8 min.

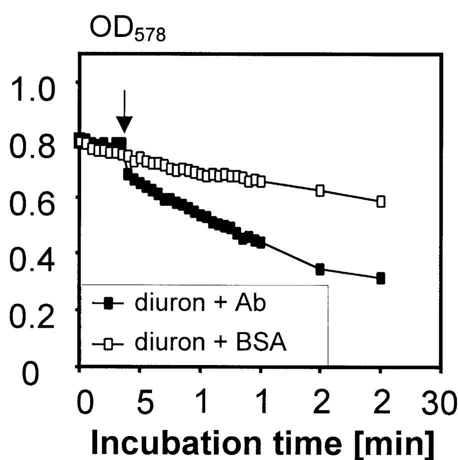

Fig. 3. Effect of the addition of antibody B91-KF5 to the diuron-inhibited Hill reaction. The assay mixtures contained 1 μM diuron at the onset of the DCPIP reduction. After 3 min, 0.5 μM antibody B91-KF5 (Ab) was added to the reaction mixtures (arrow, ■). As a control, an equimolar BSA concentration was added to the other mixture (□).

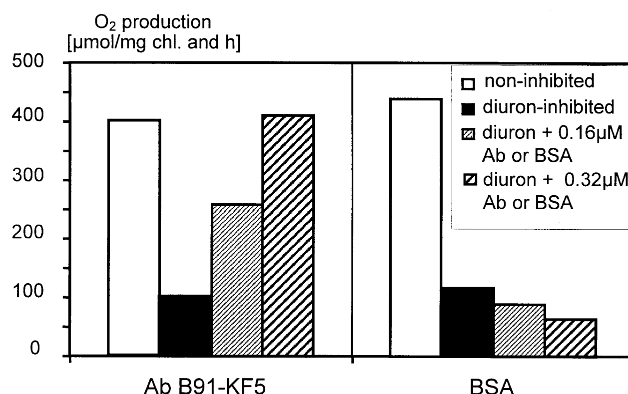

Fig. 4. Reactivation of the diuron-inhibited photosynthesis of *C. reinhardtii* Dangeard *cw15* by anti-diuron antibodies. Photosynthesis was quantified by measuring oxygen production (μmol per mg chlorophyll and h) either in the absence or the presence of 0.25 μM diuron. After 5 min, the anti-diuron antibody B91-KF5 (final concentration of 0.16 μM) was added to the diuron-containing cell suspension and the polarographic monitoring was continued. After 5 min the concentration of the monoclonal antibody was raised to 0.32 μM and the photosynthetic oxygen production was determined. As a control, photosynthesis of diuron-treated cells (0.25 μM) was quantified after the addition of equimolar concentrations of BSA.

added to the Hill reaction mixture (to give a final antibody concentration of 0.75 μM) no herbicide-dependent inhibition of the electron transport occurred. The protective effect was not observed with BSA in equimolar concentrations (Fig. 2).

In a second series of experiments the Hill reaction mixture initially contained diuron (1 μM) and, therefore, the DCPIP reduction occurred at a low rate. However, the rate immediately increased when anti-diuron antibodies were added (Fig. 3). When the Hill reaction was started with premixed diuron and BSA was then added instead of antibodies the diuron-dependent inhibition of the Hill reaction continued.

3.3. *Prevention of the herbicide-dependent inhibition of whole cell photosynthesis by anti-diuron antibodies*

Photosynthesis of the cell wall-deficient mutant of *C. reinhardtii* Dangeard *cw15* was significantly inhibited by submicromolar concentrations of diuron (Table 1). In the presence of 0.2 μM diuron the rate of the photosynthetic oxygen production was reduced by more than 50% compared to that of the control without diuron. A complete inhibition was obtained at a diuron concentration of 2 μM. Such diuron concentrations had no detectable effect on respiration in the dark (data not shown). The addition of relevant or irrelevant antibodies or BSA in the absence of diuron resulted in an approximately 20% increase in the rate of apparent photosynthesis. The reason for this stimulation of photosynthesis might be a general improvement of the growth conditions of the algae by the additional proteins.

To analyze the protective effect of the anti-diuron antibody 1 μM diuron was preincubated with either 1 or 5 μM B91-KF5 antibody for approximately 1 h at room temperature. Subsequently, an aliquot of this mixture was added to the cell suspension to give a final concentration of 0.2 mM diuron and the photosynthetic oxygen production was monitored. Under these conditions the inhibitory effect of diuron was neutralized. The inhibitory effect of diuron was retained when a control protein was used for preincubation (BSA, ovalbumin or the irrelevant monoclonal antibody B76-BF5 directed against the derivative *N*-(2-*N*-chloroacetyl-aminobenzyl)-*N'*-chlorophenyl [16]).

3.4. *Reactivation of diuron-inhibited photosynthesis of algal cells by anti-diuron antibodies*

In the presence of 0.25 μM diuron the rate of photosynthesis of *C. reinhardtii* Dangeard *cw15* diminished to approximately 25% compared to the diuron-free control. When the monoclonal anti-diuron antibody B91-KF5 was added to the reaction vessel (final concentration 0.16 μM) the rate of photosynthesis immediately increased to more than 60%. The increase of the antibody concentration to 0.32 μM resulted immediately in a completely uninhibited photosynthesis (Fig. 4). Diuron-inhibited photosynthesis was not reactivated when equimolar amounts of BSA were added to the cell suspension. Since the antibodies are unable to pass the membrane of the *Chlamydomonas* cells they have direct access only to the extra-

Table 1
Photosynthesis of *C. reinhardtii* (*cw15*) cells as affected by diuron and anti-diuron antibodies

	Reaction mixture				
Diuron (μM)	0	0.2	0	0.2	0.2
Ab B91-KF5 (μM)	0	0	0	0	0.2
Ab B76-BF5 (μM)	0	0	1	1	0
Photosynthesis (μmol O_2 (mg chlorophyll and h)$^{-1}$)	232	123	306	125	300

The photosynthetic oxygen production was monitored in the presence of varying concentrations of diuron. Monoclonal antibodies (Ab) B91-KF5 (1 μM) or B76-BF5 (5 μM) were preincubated with diuron (1 μM) for approximately 1 h at room temperature. Aliquots of the preincubation mixtures were transferred to the cell suspension to give a final diuron concentration of 0.2 μM.

Table 2
Photosynthesis of *C. reinhardtii* (*cw15*) as affected by methabenzthiazuron, monolinuron and the anti-diuron antibody (Ab) B91-KF5

Herbicides (μM)	Methabenzthiazuron			Monolinuron	
	0	2	2	1.6	1.6
Ab B91-KF5 (μM)	0	0	0.5	0	0.5
BSA (μM)	0.5	0.5	0	0.5	0
Photosynthesis (μmol O_2 (mg chlorophyll and h)$^{-1}$)	266	77	54	153	250

Following preincubation for 1 h at room temperature the antibody–herbicide mixtures were added to the cell suspension to give the final concentrations as indicated.

cellular diuron pool and can only indirectly interact with the intracellular free and D1-bound diuron. An extracellular formation of diuron–antibody complexes obviously causes an efflux of intracellular diuron which results in a release of herbicide from the Q_B site. First experiments using the cell wall-containing *C. reinhardtii* 11-32b showed that diuron-inhibited photosynthesis was also immediately reactivated by the addition of anti-diuron antibodies.

It can, therefore, be expected that an antibody produced in sufficient quantity by the plant cell itself, e.g. in transgenic plants, can also neutralize the herbicide [17]. Transgenic plants can be produced that synthesize and accumulate antibodies or antibody fragments in different compartments [18]. Since the high-affinity anti-diuron antibodies B91-KF5 and B91-CG5 effectively protect the photosynthesis against a herbicide even if they are located in the extracellular space the generation of diuron-insensitive transgenic plants is conceivable by targeting the antibodies or antibody fragments via the endoplasmic reticulum into the apoplast.

3.5. Selectivity of the protective effect of the anti-diuron antibodies

Photosynthetic oxygen production of the *Chlamydomonas* cells was also strongly inhibited by the diuron-related compounds monolinuron and methabenzthiazuron. The monoclonal anti-diuron antibody B91-KF5 inactivated monolinuron but not methabenzthiazuron (Table 2). These data are consistent with a weak cross-reactivity of the antibody with monolinuron and with the lack of cross-reactivity with methabenzthiazuron (see above). It can, therefore, be expected that transgenic plants expressing anti-herbicide antibodies are resistant to cross-reactive herbicides, too. It remains to be elucidated whether the quantity of antibodies synthesized by transgenic plants will be sufficient to neutralize a herbicide applied under field conditions.

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