A New Monoclonal Antibody for the Sensitive Detection of Atrazine with Immunoassay in Microtiter Plate and Dipstick Format†

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To develop atrazine-specific monoclonal antibodies (mAb), hybridoma cells were produced by the fusion of mouse myeloma cells (PAI-B_3AG8I) and spleen cells from mice immunized with 4-chloro-6-(ethylamino)-1,3,5-triazine-2-(6-aminohexanecarboxylic acid) coupled to keyhole limpet hemocyanin. After screening with a competitive enzyme-linked immunosorbent assay (ELISA), a mAb with a high binding affinity for atrazine was selected and used to develop a sensitive competitive direct ELISA in a microtiter plate and a dipstick format. With the microtiter plate ELISA atrazine can be determined in the range from 0.03 to 1 μg/L with a test centerpoint at 0.1 μg/L. The mAb cross-reacts predominantly with propazine (136%) and to a lower extent with cyanazine (28%) and terbuthylazine (26%). The use of a dipstick format allows the quick visual detection of atrazine in concentrations above 0.5 μg/L in aqueous samples within about 20 min.

INTRODUCTION

Atrazine is the major triazine herbicide used in corn fields worldwide. Because of its persistence and widespread application, atrazine residues cause problems as contaminants in groundwater and drinking water. In spite of its prohibition in Germany in 1991, atrazine was repeatedly detected in drinking water in concentrations above the upper limit of the directives of the European Community (EC), i.e., 0.1 μg/L. Especially for screening large numbers of samples, enzyme immunoassays are suitable tools for quick and sensitive analysis with high sample throughput (Van Emon and Lopez-Avila, 1992). In addition to the chromatographic methods currently used, immunoassays can complement the repertoire of analytical methods in residue analysis, in particular when only small sample volumes are available.

The growing interest in immunoassays leads to an increasing demand for antibodies which are able to detect the analytes in the lower parts per billion (ppb) range. Some sensitive atrazine assays using polyclonal antibodies exist (Wittmann and Hock, 1989; Dunbar et al., 1990), but often the amount of the suitable antibodies, mostly produced in rabbits, is limited, and once they are completely spent, it is difficult to raise identical antibodies. The hybridoma technique, based on the work of Köhler and Milstein (1975), makes it possible to produce an unlimited supply of antibodies with defined biological qualities. This technique has been successfully applied to the production of antibodies against atrazine and other triazines (Schlaeppi et al., 1989; Giersch and Hock, 1990; Karu et al., 1991). This paper reports on the generation of monoclonal antibodies (mAb's) for the detection of atrazine with enzyme immunoassays in concentrations well below the limits permitted by the EC guidelines for drinking water (0.1 μg/L).

The immunoassay systems used in residue analysis are mostly based on microtiter plates. They allow the quantification of analytes using sophisticated photometers, often combined with computer-automated calculation. These methods, however, generally involve several hours, and their application is normally restricted to laboratories.

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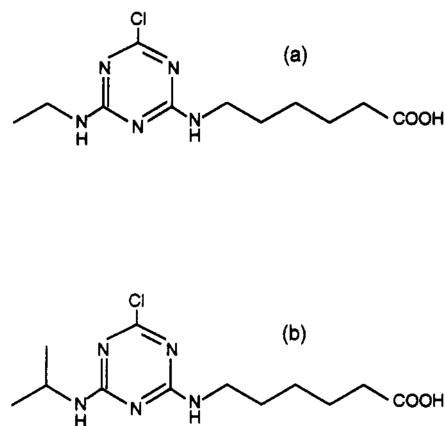

Figure 1. Schematic structure of the triazine derivative 4-chloro-6-(ethylamino)-1,3,5-triazine-2-(6-aminohexanecarboxylic acid), used for immunoconjugate synthesis (a), and of the triazine derivative 4-chloro-6-(isopropylamino)-1,3,5-triazine-2-(6-aminohexanecarboxylic acid), used for HRP tracer synthesis (b).

Dipstick or test strip assays, to date used as a diagnostic tool for monitoring drugs (Litman et al., 1983), toxins (Usleber et al., 1991; Schneider et al., 1991), hormones (Ploum et al., 1991), and pathogens, allow a rapid, qualitative determination of analytes. Our objective was to provide a comparable field test system for the screening of water samples for atrazine without expensive equipment using the mAb immobilized on a membrane support.

MATERIALS AND METHODS

Reagents. Triazine standards were kindly provided by Riedel-de Haën AG (Seelze) and Ciba-Geigy Ltd. (Basel). The triazine derivatives for immunoconjugate and tracer synthesis, 4-chloro-6-(ethylamino)-1,3,5-triazine-2-(6-aminohexanecarboxylic acid) and 4-chloro-6-(isopropylamino)-1,3,5-triazine-2-(6-aminohexanecarboxylic acid), were synthesized by Dr. U. Doht, Riedel de Haën. In addition, the following reagents were used: 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-4-toluenesulfonate (CMC; Sigma); dicyclohexylcarbodiimide (DCC; Sigma, München); keyhole limpet hemocyanin (KLH; SERVA, Heidelberg); 3,3′,5,5′-tetramethylbenzidine (TMB; Sigma); Freund's adjuvant (Sigma); horseradish peroxidase (HRP), Nutridoma-NS (Boehringer Mannheim); and goat anti-mouse IgG (No. 5899, Sigma). All other reagents were of the highest purity grade available. RPMI 1640 medium, Dutch modification (Gibco,

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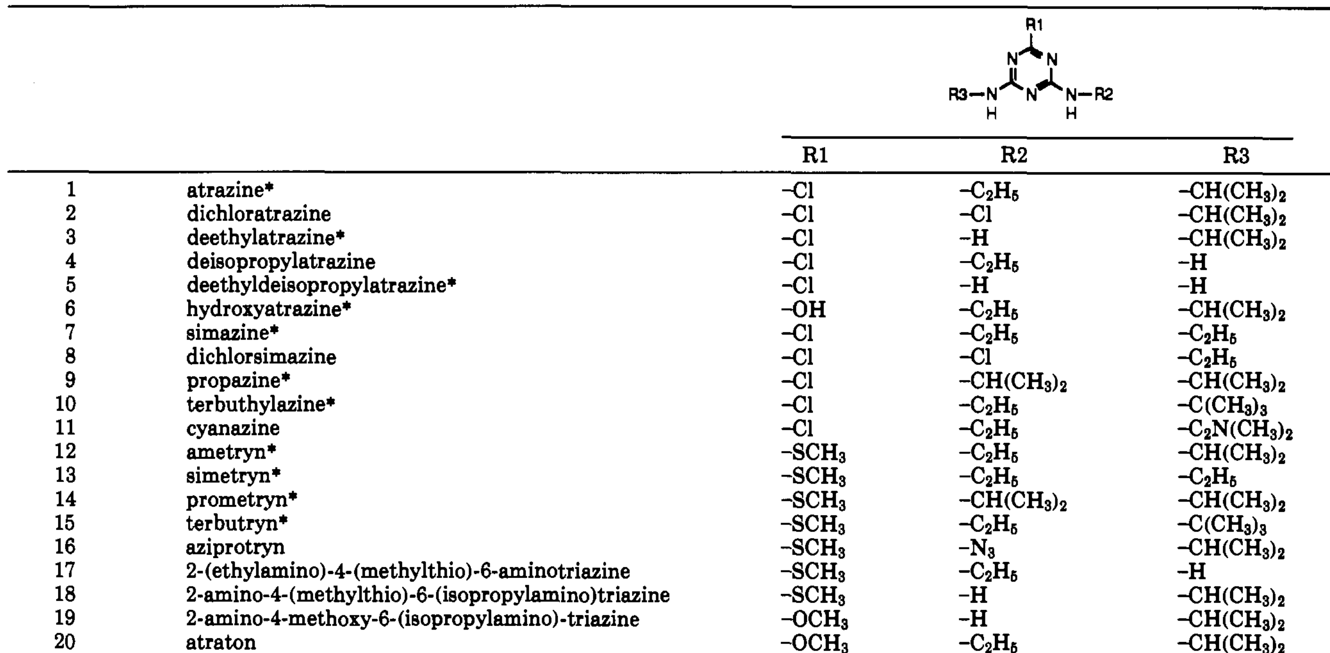
Table I. Structure and Common Names of the s-Triazines Used for Cross-Reactivity Determination[a]



		R1	R2	R3
1	atrazine*	$-Cl$	$-C_2H_5$	$-CH(CH_3)_2$
2	dichloratrazine	$-Cl$	$-Cl$	$-CH(CH_3)_2$
3	deethylatrazine*	$-Cl$	$-H$	$-CH(CH_3)_2$
4	deisopropylatrazine	$-Cl$	$-C_2H_5$	$-H$
5	deethyldeisopropylatrazine*	$-Cl$	$-H$	$-H$
6	hydroxyatrazine*	$-OH$	$-C_2H_5$	$-CH(CH_3)_2$
7	simazine*	$-Cl$	$-C_2H_5$	$-C_2H_5$
8	dichlorsimazine	$-Cl$	$-Cl$	$-C_2H_5$
9	propazine*	$-Cl$	$-CH(CH_3)_2$	$-CH(CH_3)_2$
10	terbuthylazine*	$-Cl$	$-C_2H_5$	$-C(CH_3)_3$
11	cyanazine	$-Cl$	$-C_2H_5$	$-C_2N(CH_3)_2$
12	ametryn*	$-SCH_3$	$-C_2H_5$	$-CH(CH_3)_2$
13	simetryn*	$-SCH_3$	$-C_2H_5$	$-C_2H_5$
14	prometryn*	$-SCH_3$	$-CH(CH_3)_2$	$-CH(CH_3)_2$
15	terbutryn*	$-SCH_3$	$-C_2H_5$	$-C(CH_3)_3$
16	aziprotryn	$-SCH_3$	$-N_3$	$-CH(CH_3)_2$
17	2-(ethylamino)-4-(methylthio)-6-aminotriazine	$-SCH_3$	$-C_2H_5$	$-H$
18	2-amino-4-(methylthio)-6-(isopropylamino)triazine	$-SCH_3$	$-H$	$-CH(CH_3)_2$
19	2-amino-4-methoxy-6-(isopropylamino)-triazine	$-OCH_3$	$-H$	$-CH(CH_3)_2$
20	atraton	$-OCH_3$	$-C_2H_5$	$-CH(CH_3)_2$

[a] Asterisks indicate the triazines which were used in the mixture for antibody screening.

Eggenstein), was supplemented with 10% fetal calf serum (Myoclone, Gibco), 4 mmol/L L-glutamine, and 100 mmol/L 2-mercaptoethanol. For HAT selection, hypoxanthine, aminopterin, and thymidine were added according to the procedure of Goding (1983).

Preparation of Hapten–Protein Conjugate. 4-Chloro-6-(ethylamino)-1,3,5-triazine-2-(6-aminohexanecarboxylic acid) (Figure 1a) was coupled to the carrier protein KLH by the CMC procedure: 50 mg of KLH was dissolved in 10 mL of distilled water. After the pH was adjusted to 5.5 with 0.1 mol/L HCl, 17 mg of CMC was added gradually. Twelve milligrams of the hapten dissolved in 1 mL of dimethylformamide was added dropwise to the KLH/CMC solution under constant stirring. The reaction mixture was stirred gently at room temperature for 20 h and then dialyzed exhaustively against distilled water. After determination of the protein concentration with the BCA–protein assay, the conjugate was stored in lyophilized form at 4 °C.

Synthesis of Enzyme Tracer. The triazine derivative 4-chloro-6-(isopropylamino)-1,3,5-triazine-2-(6-aminohexanecarboxylic acid) (Figure 1b) was coupled to HRP by the active ester method according to Wittmann and Hock (1989); 1 mg of the hapten together with 1.7 mg (=15 μmol) of N-hydroxysuccinimide and 6.2 mg (=30 μmol) of dicyclohexylcarbodiimide was dissolved in 130 μL of dry dioxane. After incubation overnight at 20 °C, the reaction mixture was slowly added to a solution of 2 mg of HRP (1350 units/mg = 22 500 nkat) dissolved in 3 mL of sodium hydrogen carbonate (0.13 mol/L) and stirred for another 3 h. Unbound hapten was removed by gel filtration on a Sephadex G-25 column (1 × 5 cm) equilibrated with PBS (phosphate buffer, 50 mmol/L, pH 7.2, 8% saline). The HRP tracer could be stored at 4 °C for more than 1 year without loss of activity. Another tracer with the triazine derivitive used in immunoconjugate preparation (Figure 1a) was synthesized accordingly.

Immunization and Fusion Protocol. Four female BALB/c mice 12–24 weeks old were intraperitoneally injected with 20 μg of hapten–KLH–conjugate in 200 μL of saline (0.9 og NaCl/L), which was emulsified 1:1 in Freund's complete adjuvant. Two injections with Freund's incomplete adjuvant followed at 4-week intervals. Serum titers were measured by ELISA 1 week after the last injection. After a rest period of 4–5 weeks, the mice with high serum titers of triazine-specific antibodies received daily booster injections with 400 μg of KLH–hapten conjugate in 200 μL of saline on the 4 days prior to the cell fusion according to the method of Stähli et al. (1980). On the fifth day the mice were sacrificed and the immune spleen cells were fused with the murine myeloma cells (PAI-B_3A8I) at a ratio of 2:1 using PEG 4000 as described previously (Giersch and Hock, 1990). Following fusion, one-fifth of the cells was seeded immediately into four 96-well culture plates. The remaining cells were frozen for a later screening. After a 2-week HAT selection period, the supernatants from wells with growing hybridomas were assayed for triazine-specific antibodies by a competitive enzyme immunoassay (ELISA).

Evaluation of Fusion Products with ELISA. Flat-bottom polystyrene microtiter plates (Greiner, Nürtingen) were precoated overnight with 250 μL/well of goat-anti mouse IgG (5 μg/mL carbonate buffer: $NaHCO_3/Na_2CO_3$ 50 mmol/L, pH 9.6) at 4 °C. The plates were drained and stored frozen at −24 °C or used immediately as follows. After a washing step with PBS–Tween 20 (5 mmol/L PBS supplemented with 0.05% Tween 20), the culture media were transferred in duplicate (100 μL/well) to the microtiter plate. Then 100 μL of PBS was added to each well. After incubation at room temperature for 2 h or at 4 °C overnight, unbound antibodies were washed off with PBS–Tween 20. Aliquots of 150 μL of PBS or PBS containing a mixture of 11 triazines (1 μg/L each) (Table I) was added in duplicate to the hybridoma–medium-coated wells. This was followed by the addition of 50 μL of HRP-labeled triazine in PBS. After a 60-min incubation, the plates were washed and 200 μL/well TMB substrate was added. The TMB substrate consisted of two parts of phosphate buffer (140 mmol/L, pH 5.0) containing 3 mmol of urea peroxide and one part of 1.2 mmol/L TMB in 8 mmol/L phosphoric acid containing 10% dimethyl sulfoxide and 12 mg/L penicillin G. The substrate reaction was stopped after 30 min with 50 μL of H_2SO_4 (4 mol/L), and the absorbance was measured at 450 nm with an ELISA reader (Multiscan MK II, ICN-Flow, Meckenheim).

Hybridoma cultures that proved to be positive in the ELISA were cloned by single-cell deposition in 96-well plates under visual control. Clones were retested by ELISA, and positives were expanded on 24-well culture plates for further characterization. Clones with the lowest detection limit for atrazine in an ELISA were recloned to ensure population uniformity and were then expanded for antibody production. Aliquots of the cultures were cryopreserved in RPMI 1640 culture medium containing 12% dimethyl sulfoxide and 20% FCS and stored in liquid nitrogen.

Monoclonal Antibody Production and Purification. The cell clone K4E7 was adapted to serum-free medium [DMEM/F12 medium with stable glutamine (Biochrom, Berlin) supplemented with Nutridoma-NS as serum replacement] and grown in roller bottles or Teflon cell culture bags (AFC, Silver Spring, MD). The mAb's from the serum-free culture supernatants were

concentrated and purified in one step by affinity chromatography using Spectra/Gel Fast-IgG (Spectrum, Houston, TX). After subsequent buffer exchange and additional concentration by ultrafiltration, the mAb's were aliquoted and lyophilized.

Characterization of Monoclonal Antibodies. The isotype of the antibodies was determined with a double-sandwich ELISA using rabbit antibodies directed against the mouse subclasses IgG1, IgG 2a, IgG 2b, IgA, IgM, and the k and l light chain (Biorad, München).

Microtiter Plate ELISA for Atrazine. The microtiter plate assay was performed as described above for the antibody screening procedure after the antibody and tracer concentrations were optimized by checkerboard titration. Data analysis was performed with the aid of a commercial ELISA software package (EIA 3, ICN-Flow, Meckenheim) using a four-parameter logistic equation for curve fitting and calculating the atrazine concentrations of samples.

Determination of Antibody Specificity. The cross-reactivities of the selected mAb's were determined with 20 triazines and several non-triazine pesticides (Table I). Calibration curves in a range from 0.01 to 1000 μg/L were employed in the competitive ELISA. After the data were normalized by the % B/B_0 transformation (eq 1, where A is the absorbance, A_0 is the absorbance at zero dose of hapten, and A_{xs} is the absorbance at an excess of hapten), the molar triazine concentrations that caused 50% inhibition were used to calculate cross-reactivities according to eq 2.

$$\% B/B_0 = (A - A_{xs}/A_0 - A_{xs}) \times 100 \quad (1)$$

$$\% \text{ cross-reactivity} = \frac{\text{mol of atrazine at } 50\% B/B_0}{\text{mol of cross-reacting triazine at } 50\% B/B_0} \times 100 \quad (2)$$

Water Sample Analysis by ELISA. Samples of tap water (Freising, Germany, not chlorinated), surface water from a pond (cleared by centrifugation), and rain water collected near Freising were spiked with standard solutions of atrazine and analyzed with ELISA to detect the influence of different matrices on the test system. Standards and samples were run in quadruplicate on the microtiter plate and repeated three times on different plates.

Dipstick Immunoassay. Preparation of the Dipsticks. To prepare the test strips, nitrocellulose (NC) (Sartorius no. 11336-41BL) or Immunodyne (Pall GmbH, Dreieich, Germany) membranes (5 × 100 mm) were coated with goat anti-mouse IgG (5 μg/mL carbonate buffer: $NaHCO_3/Na_2CO_3$, 50 mmol/L, pH 9.6) for 4 h at room temperature on a horizontal shaker. After being washed with PBS-washing buffer, the strips were dried at room temperature and mounted onto the border of plastic supports using double-sided adhesive tape. In the next step, drops of the mAb K4E7 (2 μL, ca. 1 μg IgG/mL PBS) were spotted on the membrane 5 mm apart. After a 30-min drying period, residual binding sites on the membrane were blocked by immersing the membrane in PBS containing 2% w/v sodium caseinate for 30 min. After washing and drying, the plastic sheet was cut into 0.5 × 5 cm strips, each carrying the coated membrane on one end. At this stage the test sticks were ready to use in the assay and could be stored at 4 °C for several months.

Substrates for the Membrane Assay. A TMB substrate (Lauritzen and Lindhardt, 1988) was modified to obtain two stable components which were ready to use after mixing: solution A, 1 mL of 7.2% TMB in dimethyl sulfoxide mixed with a solution of 0.8% dioctylsulfosuccinate in EtOH; solution B, sodium acetate (50 mmol/L) containing 0.015% urea peroxide. Before use, 1 part of solution A was mixed with 4 parts of solution B. This substrate system was compared with other HRP substrates suitable for membrane assays: 4-chloronaphthol (CN), diaminobenzidine (DAB), and a mixture of both (CN/DAB), all prepared according to the methods of a Young (1989), and the commercially available detection system IBI Enzygraphic web (Kodak, New Haven) based on a coated polymer film.

Assay Protocol. To perform the assay, 1 mL of the sample solution, atrazine standards, or atrazine-free control solutions was mixed with 50 μL of HRP tracer at the appropriate concentrations in a test tube.

The dipsticks were incubated in these mixtures for 5 min at room temperature with gentle agitation. After a washing step, the test strips were developed in the substrate solution for 10 min. The color intensity of the spots was visually compared with the negative controls, which showed the most intense color development. The atrazine concentration at which the color intensity could be clearly distinguished from the negative control by several persons was considered to be the detection limit.

RESULTS AND DISCUSSION

MAb Production and Characterization. The spleen cells from the mouse exhibiting the highest serum titer after immunization with the 4-chloro-6-(ethylamino)-1,3,5-triazine-2-(6-aminohexanecarboxylic acid)–KLH conjugate were fused with the myeloma cell line PAI-B_3Ag8I. Growing hybridomas were observed in more than 90% of the wells. A fusion frequency of 10^{-5} was calculated on the basis of the cells employed in cell fusion. The first seeding resulted in 35 triazine-positive clones; i.e., the absorbances of the control wells were more than twice the absorbance values of the corresponding wells with the triazine mixture. After subcloning, the clone K4E7, which exhibited high affinity for atrazine, was chosen for immunoassay development in microtiter plates and a dipstick assay based on antibody-coated membranes. The isotype of the mAb is IgG2b with kappa light chains.

Antibodies were produced on a laboratory scale using gas-permeable Teflon bags or roller bottles. In both systems, cell concentrations of more than 10^6 cells/mL were reached with this clone using serum-free media. The antibody concentrations varied between 50 and 100 μg/mL depending on cell concentration and medium-changing intervals.

The crude cell-culture supernatants could be used directly in the ELISA, but a purificaiton of the mAb was preferred for longer storage and reagent standardization. In contrast to protein A agarose matrices, which require preconcentration and buffer exchange before binding, the Spectra/Gel fast-IgG matrix binds the immunoglobulins directly from cell-culture supernatants. This allows the one-step purification and concentration of the mAbs from the cell-culture medium. For use in ELISA the lyophilized mAb was reconstituted with PBS to a concentration of 10 mg/mL. This stock solution was used for further assay optimization. Checkerboard titration resulted in an optimum mAb dilution of 1:00000 corresponding to a concentration of about 10 ng/mL using goat anti-mouse IgG coated plates.

Microtiter Plate Assay. The choice of the right enzyme tracer often has a considerable influence on the performance of an immunoassay (Ulrich et al., 1991; Weller et al., 1992). When two different enzyme tracers carrying either an ethyl or an isopropyl group as alkyl substituent were compared in an ELISA, nearly identical standard curves were observed. This indicates that in the case of these substituents no effect of heterology between immunoconjugate and tracer occurs. All measurements described here were performed using the enzyme tracer carrying the triazine with an isopropyl derivative in a dilution of 1:50000. The use of anti-mouse IgG coating for trapping the mAb gave the best performance in triazine immunoassays with the mAb. In addition, it allows an economical use of antibodies. Similar observations were made by Schneider and Hammock (1992) and by Giersch et al. (1992).

A typical standard curve of the microtiter plate ELISA, shown in Figure 2, exhibits a 50% binding value of approximately 0.1 μg/L. The minimum detectable amount of atrazine, defined as 80% B/B_0, was 0.03 μg/L. This

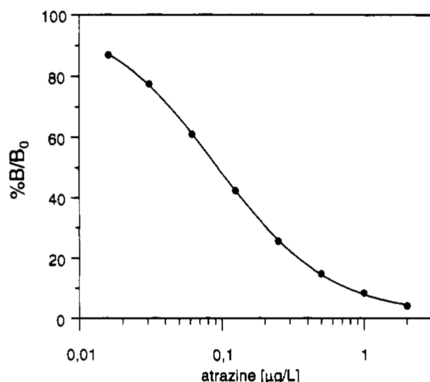


Figure 2. ELISA standard curve for atrazine with mAb K4E7 (absorbance value of zero control = 0.9).

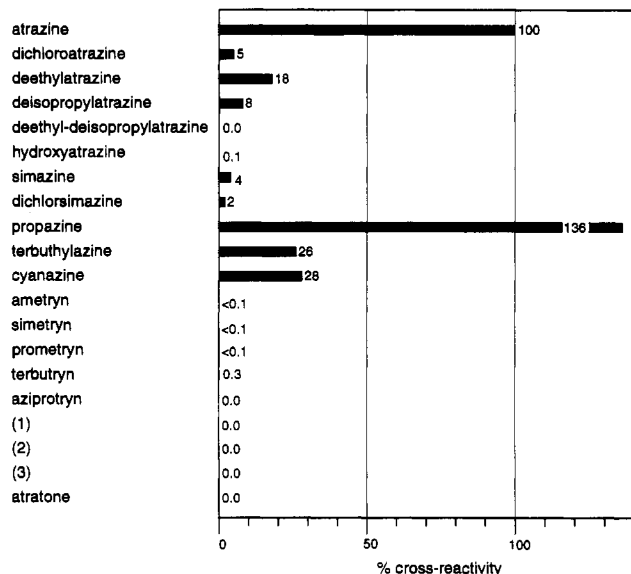


Figure 3. Cross-reactivity pattern of mAb K4E7 with different triazines. (1) 2-(Ethylamino)-4-(methylthio)-6-aminotriazine; (2) 2-amino-4-(methylthio)-6-(isopropylamino)triazine; (3) 2-amino-4-methoxy-6-(isopropylamino)triazine. The following non-triazine pesticides showed no reactivity in concentrations up to 1000 µg/L: 3-amino-1H-1,2,4-triazole, methabenzthiazuron, metazachlor, metribuzin, permethrin, propiconazol, and prochloraz.

means that atrazine levels near the limit of the European drinking water regulation (0.1 µg/L for a single substance) correspond best with the highest precision of the test system described here.

The cross-reactivity measurements based upon atrazine (100%) are shown in Figure 3. Although the immunoconjugate carries a free chlorine and an ethyl group, the highest affinities were observed with derivatives with one (atrazine) or two (propazine) isopropyl groups. This common feature of most atrazine-sensitive antibodies may be caused by the alkyl-chain spacers introduced in coupling reactions. The spacer may mimic alkyl groups such as isopropyl and stimulate the corresponding B-lymphocytes during immunization. Additionally, the chlorine group seems to be essential for this antibody for good hapten recognition, because no reactivity or only extremely weak reactivity was observed with triazines lacking the chlorine group. In contrast, mAb's against triazines produced by immunization with sulfur-containing haptens show better recognition of the "triatrynes" (Giersch and Hock, 1990; Karu et al., 1991).

Regardless of which derivative is used for conjugate preparation, the immunization stimulates a lot of B cells exhibiting various specificities for the different triazines. While screening the fusion products of these cells, clones may be selected which produce antibodies not exactly fitting the hapten used for immunoconjugate synthesis. Similar observations were made in the case of an mAb with high specificity for terbutylazine (Giersch et al., 1993). The high sensitivity of some polyclonal immunoassays for an analyte often depends on a small group of high-affinity antibodies without the whole polyclonal mixture. The development of monoclonal antibody-based immunoassays with low detection limits depends on the selection of such clones secreting antibodies with very high affinities. Work is in progress to shorten and to improve this time-consuming step by using immunomagnetic cell separation (Hock et al., 1992).

The high cross-reactivity to propazine exhibited by mAb K4E7 seems to be an inherent property of all antibodies with a high sensitivity for atrazine. Cross-reactivities which often exceeded 100% could be observed in most polyclonal (Wittman and Hock, 1989; Dunbar et al., 1990; Wüst and Hock, 1992) and monoclonal antibodies (Schlaeppi et al., 1989; Karu et al., 1991). However, this does not affect sample measurements in regions with limited application of propazine such as western Europe (except Belgium and The Netherlands) and the United States.

Table II. Detection of Atrazine in Spiked Water Samples by ELISA[a]

spike	detected		
	tap water[b]	pond water[c]	rain water[d]
0	0.013 ± 0.01	0.050 ± 0.01	0.052 ± 0.01
0.1	0.105 ± 0.01	0.131 ± 0.01	0.164 ± 0.01
0.5	0.501 ± 0.20	0.497 ± 0.02	0.530 ± 0.02

[a] All values are given in micrograms per liter. All samples were analyzed undiluted in quadruplicate; are means with standard deviations are given. [b] Collected in Freising in Sept 1992. [c] Freising, Aug 1992. [d] Collected near Freising in Sept 1992.

To detect potential matrix effects in drinking, rain, and surface water, samples from different sources were spiked with defined amounts of atrazine and examined with the optimized ELISA. Representative results are shown in Table II. No water samples tested to date show any influence of the different matrices worth mentioning. The test system will now be applied to the screening of soil samples and samples of free soil water, collected by use of suction lysimeters.

Dipstick Assay. Dipstick assays, so far used to detect drugs, hormones, and toxins, could also be a valuable tool for the quick detection of pesticide contaminants in water and soil without expensive equipment. To develop a quick field test in a dipstick format for determining atrazine, the immunoassay was adapted to a membrane solid phase mounted onto a plastic support. Of the two membranes tested, the NC membrane exhibited a higher signal to noise ratio than the Immunodyne membrane and was selected for the test system. With both membrane types, the precoating step with anti-mouse IgG resulted in good reproducibility and stability of the test strips. The dried strips, which were stored in a refrigerator, showed no deterioration in quality over a period of 8 months.

The detection limit of a competitive immunoassay is correlated with the dilution of the enzyme-labeled tracer. Therefore, the ability of a substrate system to make low concentrations of peroxidase visible considerably influences the performance of a membrane assay. To compare different substrates with respect to sensitivity and easy applicability in the dipstick assay, the mAb-coated dip-

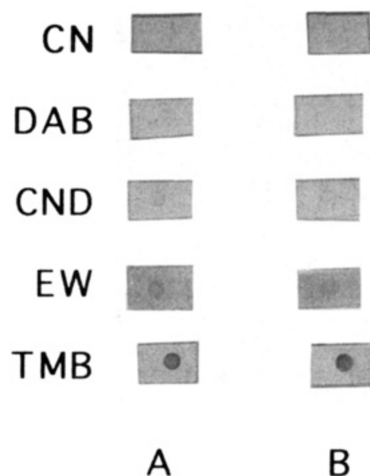


Figure 4. Comparison of the signal of different substrate systems: CN, 4-chloronaphthol; DAB, diaminobenzidine; CND, mixture of both; EW, Enzygraphic web; TMB, tetramethylbenzidine. Tracer dilutions: (A) 1:20000; (B) 1:40000.

sticks were incubated with HRP tracer in working dilutions of 1:20000 and 1:40000. Figure 4 shows the color development of different substrates. The chromogens DAB, CN, and the optimized mixture CN/DAB were used according to the method of Young (1989). With DAB and CN no signal was visible at these tracer concentrations. The mixture gave only a faint color development with the higher tracer concentration. These classical substrates, often used for HRP visualization on blots, are not suited to detecting the low tracer concentrations necessary for a sensitive competitive immunoassay on membranes.

TMB is a commonly used soluble chromogen for microtiter plate ELISAs. The system of Lauritzen and Lindhardt (1988) allows the use of TMB as a precipitating chromogen for HRP detection on membranes. Our modification resulted in two components ready for use which were stable for several months at 4 °C. The storage of the two components at room temperature for several days caused no loss in activity. This stability without freezing is an advantage, especially for use in field applications. The signal of this substrate was clearly visible at higher tracer concentration (Figure 4A), and it could be differentiated from the background even at the lower concentration of the HRP tracer.

The ready-to-use HRP detection system IBI Enzygraphic web (EW) showed a signal somewhat weaker than the signal of the TMB substrate with higher background noise. The handling of the EW, conceived especially for larger blotting membranes, was not as easy as that of liquid substrates, because the sheets had to be cut in small pieces for color development on single dipsticks. Due to the recommendation of the manufacturer to keep the EW frozen until use, its application is limited to the laboratory.

The modified TMB system turned out to be the best substrate with regard to sensitivity, stability, and ease of handling and was used throughout further optimization and application of the dipstick assay.

The dipstick assay allows a semiquantitative determination of atrazine in aqueous solutions, e.g., the determination of a lower limit by visual evaluation. As dot color intensity is inversely related to the atrazine concentration, the visual detection limit is the concentration of atrazine that results in a dipstick color intensity clearly distinguishable from the signal of the negative control. Concentrations of antibody and tracers were optimized at different incubation times to result in a clear difference at low atrazine doses. An incubation time of 5 min in the competition step was chosen as the optimum. A prolonged incubation time did not intensify the color, whereas a shorter time resulted in a weaker signal. Using these parameters, the dipstick assay is able to indicate atrazine above the level of 0.5 μg/L in different water samples as a clear reduction in color development compared to the control (Figure 5). At concentrations above 1 μg/L color development was completely suppressed. Atrazine concentrations of 0.1 μg/L resulted in most cases in a slight but distinguishable difference compared to the negative control. In some cases the difference at the 0.1 μg/L level was difficult to distinguish. This can be attributed to variations in the unautomated coating and incubation steps. The 0.5 μg/L level was chosen as the detection limit because the test system is considered to be a field test, which should give clear-cut results even under nonoptimal conditions. By reducing the tracer concentration, a lower detection limit can be attained at the expense of the control signal. Because comparable dipstick assays for triazines or other pesticides have not yet been published, dipstick assays from other fields have to be used by way of comparison. Assays described for residue determination of different toxins (Schneider et al., 1991; Usleber et al., 1991) and hormones (Ploum et al., 1991) require sample incubation times of at least 30 min. The short sample incubation time sufficient for reaching an optimal signal in the atrazine assay indicates high association rates of mAb K4E7. Compared to the corresponding microwell plate ELISAs, all dipstick assays achieve equilibrium of the specific immunoreaction much faster. The detection limit for atrazine achieved with the assay described here corresponds to the most sensitive assays for mycotoxins, which are in the range 0.2–15 μg/L (Schneider et al., 1991). The assays for the hormones clenbuterol and β-nortestosterone reach detection limits between 1 and 5 μg/L (Ploum et al., 1991). A quantitative determination of atrazine in the field should be possible by instrumental evaluation using reflection measurement, e.g., by small pocket reflectometers. In addition, the detection limit may be lowered due to the better differentiation between atrazine-positive test strips and the negative control.

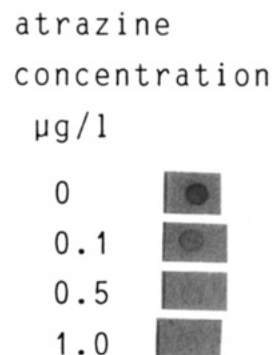


Figure 5. Results of a dipstick assay with water containing different atrazine concentrations.

Conclusions. The development of new mAb's for the determination of triazines such as atrazine in addition to existing antibodies will help to improve the performance of ELISAs in terms of detection limit and in providing immunoreagents with defined specificities over a long period of time. Both qualities are necessary for a wider acceptance in residence analysis. Moreover, the availability of a panel of well-characterized mAb's with different specificities may make the identification of different triazines with immunoassays possible as recently shown by Schneider et al. (1992).

Besides the microtiter plate format ELISA, a rapid noninstrumental dipstick immunoassay for atrazine was developed giving semiquantitative results within less then 20 min. The distinction between positive and negative atrazine samples can be made at approximately 0.5 μg/L. The test system is a valuable tool for the rapid detection of atrazine in water samples in the field, e.g., to prove the illicit application of atrazine or to exclude the risk of residue problems in the rotation of crops.

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