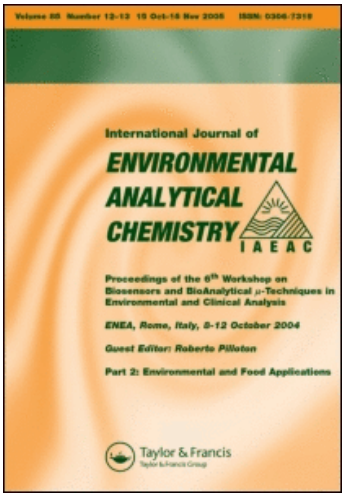

International Journal of Environmental Analytical Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713640455

Monoclonal Antibodies Useful in Sensitive Immunoassays for Aldicarb in Either Laboratory or the Field

Lai Khai Siew[a]; Larry A. Winger[a]; Julia A. Spoors[a]; John L. Dessi[a]; Lyn Jennens[b]; Colin H. Self[ac]
[a] Department of Clinical Biochemistry The Medical School, University of Newcastle, Newcastle upon Tyne, UK [b] Formerly of Aventis CropScience, Ongar, Essex, UK [c] Selective Antibodies Ltd, Ponteland, Northumberland, UK

Online publication date: 17 September 2010

To cite this Article Siew, Lai Khai , Winger, Larry A. , Spoors, Julia A. , Dessi, John L. , Jennens, Lyn and Self, Colin H.(2003) 'Monoclonal Antibodies Useful in Sensitive Immunoassays for Aldicarb in Either Laboratory or the Field', International Journal of Environmental Analytical Chemistry, 83: 5, 417 — 426
To link to this Article: DOI: 10.1080/0306731031000099819
URL: http://dx.doi.org/10.1080/0306731031000099819

Intern. J. Environ. Anal. Chem., Vol. 83, No. 5, pp. 417–426

Taylor & Francis
Taylor & Francis Group

MONOCLONAL ANTIBODIES USEFUL IN SENSITIVE IMMUNOASSAYS FOR ALDICARB IN EITHER LABORATORY OR THE FIELD

LAI KHAI SIEW[a], LARRY A. WINGER[a], JULIA A. SPOORS[a], JOHN L. DESSI[a], LYN JENNENS[b] and COLIN H. SELF[a,c,*]

[a]*University of Newcastle, Department of Clinical Biochemistry, The Medical School, Framlington Place, Newcastle upon Tyne, NE2 4HH, UK;*
[b]*Formerly of Aventis CropScience, Fyfield Road, Ongar, Essex, CM5 0HW, UK;*
[c]*Selective Antibodies Ltd, Little Callerton House, Runnymede Road, Ponteland, Northumberland, NE20 9HJ, UK*

(Received 25 February 2002; in final form 27 May 2002)

Although polyclonal antisera have been used for immunodiagnostic measurement of aldicarb, both as components of commercial kits and as stand-alone products, no monoclonal antibody (MAb) reactive to this carbamate pesticide has yet been described. We have generated two MAbs with specificity to aldicarb, which function in our prototypic field immunoassays to identify this chemical in water samples. Further exploitation of these inherently stable immunoreagents should be valuable for point-of-need testing for the presence of aldicarb.

Keywords: Aldicarb; Immunoassay; Monoclonal antibody; Carbamate; Pesticide

INTRODUCTION

Aldicarb, a carbamate pesticide used against insects, mites and nematodes in field crops, vegetables, fruits and ornamental plants [1], is also the major constituent of the slug pellets placed extensively in domestic gardens in the UK. The American Environmental Protection Agency classifies aldicarb in its highest category of toxicity [2]. Persistence of aldicarb in shallow ground wells has been documented [3] thus making regular and convenient monitoring for aldicarb residue in ground water or indeed food [4] worthwhile.

Monitoring of aldicarb in ground water is not a trivial matter, though conventionally, the monitoring process is achieved by physical techniques such as gas chromatography (GC) and high pressure liquid chromatography (HPLC) [5]. These techniques are labour intensive; moreover, they are neither inexpensive nor mobile.

*Corresponding author. Fax: +44(0)191-222-6227. E-mail: C.H.Self@ncl.ac.uk

ISSN 0306-7319 print: ISSN 1029-0397 online © 2003 Taylor & Francis Ltd
DOI: 10.1080/0306731031000099819

Immunoassays, on the other hand, can certainly offer some advantages over the conventional methods for the analysis of pesticides. They are selective, sensitive and simple to use. Hence they offer a cheaper and faster alternative monitoring system to identify the presence of a targetted pesticide and to flag positive samples for later confirmation by physical procedures [6]. A commercially available immunoassay system, EnviroGard Aldicarb Plate Kit, uses polyclonal antisera, and exhibits sensitivities in the region of 1 ppb (ng/mL or μg/L). Alternatively, laboratories have been able to use commercially available sheep anti-aldicarb antisera (BioDesign International, Kennebunkport, ME) to construct their own immunoassays.

However, immunoassays based on polyclonal antisera are always subject to intrinsic batch-to-batch variation, dependent on the finite pool of serum used to prepare the kit. Monoclonal antibodies (MAbs) have replaced polyclonal antisera in many clinical diagnostic assays not least for reasons of stability and consistency.

We report here the generation of two different MAbs recognising aldicarb, which may be useful in immunoassays based either in the analytical laboratory, or in the field. As enzyme substrates e.g. the alkaline phosphatase amplification system [7] now formated as Ampli-Q (DAKO), or the new Attophos substrate (JBL Scientific) – continue to improve, such quantitative immunoassays might be configured to exhibit more enhanced sensitivity, with commensurately better results in flagging positive samples. On the other hand, if appropriate sensitivity levels can be achieved, the lateral flow technologies may provide convenient point-of-need tests.

EXPERIMENTAL

Aldicarb Analogue Preparation

An aldicarb analogue with a carboxylic acid residue was constructed in the laboratories of Aventis CropScience, Ongar, UK according to the scheme depicted in Fig. 1.

Essentially, aldicarb oxime was converted to an ester derivative, by reaction with ethyl isocyanoacetate. The ester derivative was then transformed to a carboxylic acid under basic conditions. This carboxylic acid was used as the aldicarb analogue for conjugation procedures.

Preparation of the aldicarb analogue began with 'Aldicarb oxime', a gift from Rhone-Poulenc Agriculture Limited, Research Triangle Park, Durham, North Carolina, USA. All reagents used in the preparation of the aldicarb analogue were 'Reagent Grade' or dry reagents (e.g. in 'Sure-Seal' bottles) from either Sigma-Aldrich, Aldrich or Lancaster suppliers.

To prepare aldicarb acetate ethyl ester, a solution of ethyl isocyanatoacetate (1.94 g, 0.015 moles, 1.0 equivalents) in dry dichloromethane (4 mL) was added to a cooled (10°C) solution of aldicarb oxime (2.0 g, 0.015 moles, 1.0 equivalents) and dry triethylamine (0.5 mL) in dichloromethane (30 mL), dropwise. The solution was stirred at ambient temperature for 1 h, then heated to 40–45°C for 1 h. The cooled solution was partitioned between water (40 mL) and dichloromethane (20 mL); the organic phase was separated and washed with further water (2×20 mL), dried (magnesium sulfate) and concentrated to give the ester as a white solid, 3.54 g, 90% crude yield as determined by NMR (Nuclear Magnetic Resonance) and LCMS (liquid chromatography–mass spectrometry).

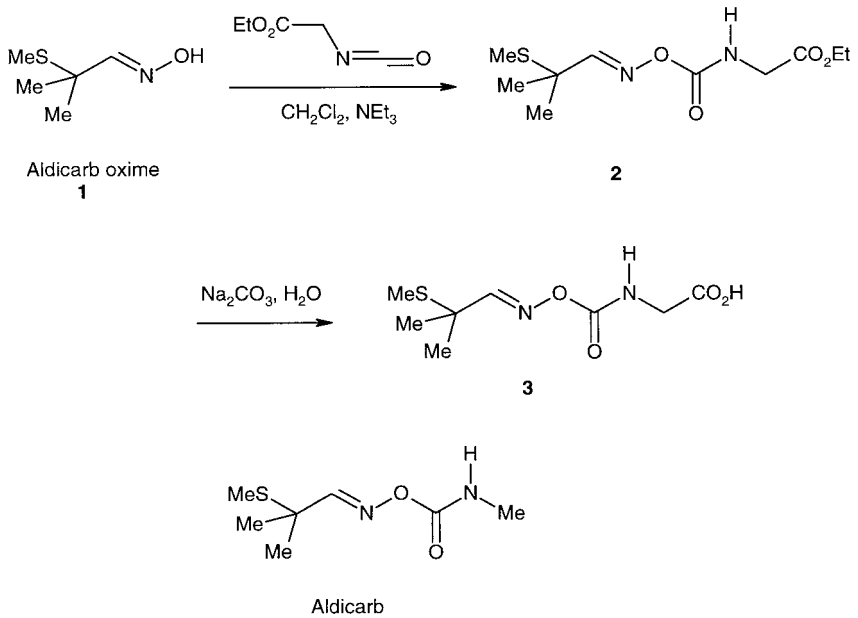

FIGURE 1 Schematic diagram illustrating the synthesis, for protein-conjugation purposes, of an aldicarb analogue from aldicarb oxime bearing a carboxylic acid terminus. Aldicarb oxime **1** was converted to the ester derivative **2**, by reaction with ethyl isocyanoacetate. The ester **2** was then transformed to the acid **3** under basic conditions. This carboxylic acid was used as the aldicarb analogue for conjugation procedures.

This material was hydrolysed directly; aldicarb acetate ethyl ester (3.54 g, 0.0135 moles, 1.0 equivalents) was added to an aqueous solution of sodium carbonate (10 g, 0.094 moles, 7 equivalents; in 300 mL water). Methanol (10 mL) was added to effect solution and the mixture stirred at 55°C for 30 min. The solution was allowed to cool to ambient temperature and the organic solvent removed *in vacuo* (rotary evaporator). The remaining aqueous material was taken to pH 3 with concentrated hydrochloric acid and extracted with ethyl acetate (3×100 mL). The combined organic phases were dried (magnesium sulfate) and concentrated, affording 2 g yellow oil. Analysis was by infra-red spectroscopy (showing the functional groups present) and NMR, LCMS as above, showing a molecular ion of 234 for the product. This material was pure enough to use in conjugation.

Hapten–Protein Conjugations

Aldicarb and aldicarb–protein conjugates (Key-hole Limpet Haemocyanin, KLH; Alkaline Phosphatase, ALP; Bovine Serum Albumin, BSA) were produced in the laboratories of Aventis CropScience, Ongar, UK. The aldicarb analogue was conjugated to KLH or to ALP by the mixed anhydride method [8]. Briefly, triethylamine and isobutyl chloroformate were added sequentially to a solution of the carboxylic acid in tetrahydrofuran, at 0°C and the mixture stirred for 30 min at this temperature. The resulting suspension was added drop wise to a cooled (4°C) solution of KLH in aqueous pyridine and the mixture stirred at 4°C for 1 h, then at ambient temperature for 1 h. The mixture was dialysed over 3 days against 3×2 L of PBS, phosphate buffered saline, (pH 7.2) at 4°C, using Visking tubing, molecular cut off 12 KDa.

For the aldicarb–BSA conjugate, we used the carbodiimide water soluble method following standard recipes [8]. Briefly, 1-ethyl-3-(dimethylaminopropyl) carbodiimide · hydrochloric acid (HCl) was added in small portions to a solution of the carboxylic acid in 25% ethanol in water and the mixture stirred for 5 min. A solution of BSA in 25% ethanol in water was added slowly to the acid/EDPC adduct and the pH of the mixture adjusted to 7.0 with aqueous 0.1 M sodium hydroxide. The mixture was stirred in the dark at ambient temperature for 24 h, then dialysed over three days against 3×5 L of PBS (pH 7.2) at 4°C, using Visking tubing impermeable to molecules larger than 12 kDa.

Miscellaneous Reagents

All chemical reagents and culture medium, except where specified, were purchased from Sigma, UK.

Immunisation, Serological Assay and Hybridoma Construction

Eight Balb/c mice were initially immunised with the immunogen, aldicarb–KLH in Freund's Complete Adjuant (1 : 1, v : v) subcautaneously in two sites at 0.1 mL (50μg) per site. They were subsequently boosted by subcutaneous route using immunogen mixed with Freund's Incomplete Adjuvant at three weekly intervals over four months.

After 2 and again after 3 immunisations, a test bleed for each mouse was taken. The immune responses were monitored by means of ELISAs (enzyme linked immunosorbent assays) using hapten conjugated to a different protein conjugate, i.e. aldicarb–BSA.

Flat bottom polystyrene 96 well Immulon-I plates (Dynex) were coated with 100 μL of a 5 μg/mL aldicarb-BSA solution in 0.05 M carbonate buffer, pH 9.6, overnight at 4°C. The plates were blocked with 100 μL of 0.5% w/v BSA in carbonate buffer for 1 h at room temperature, and then washed in Tris–HCl/Trition (T/T), 3 times per wash cycle. A 50 μL aliquot of TT containing 0.2% BSA w/v (TBT), with or without aldicarb at 1000 ng/mL was added to the wells. Sequentially a 50 μL aliquot of the immune sera diluted in TBT was added to the wells. The mixture was incubated in the wells for 1 h at room temperature, washed and a 100 μL aliquot of ALP-labelled Goat anti-Mouse IgG Fc at 1 : 1000 in TBT was added to the wells. After 1 h incubation at room temperature, the plates were washed and developed with *p*-nitrophenyl phosphate substrate. The results were quantified with an ELISA plate reader (Parkard Argus 300) at 405 nm.

Aldicarb-specific responders, after 3 immunisations of 50 μg immunogen, were identified and four days before fusion, were given a final intra-splenic dose of immunogen in PBS.

Splenocytes from these mice were fused with the mouse myeloma line, NS-l, using the conventional hybridoma protocol as described [9]. The resultant hybridomas were cultured in RPMI 1640 selective medium containing hypoxanthine, aminopterin and thymidine, and 10% foetal calf serum. Ten days after the fusion, the cultures were screened for growth and subsequently for positive reactivity with the aldicarb–BSA hapten–protein conjugate, with or without aldicarb. Wells exhibiting reactivity to the hapten–protein conjugate, which were in addition sensitive to the addition of hapten, were re-tested to confirm this hapten-specific reaction. Positive colonies were subcloned

several times at 0.3 cells/well to ensure monoclonality before expansion in tissue culture for further production and analysis.

Two MAbs binding to aldicarb were identified. These MAbs were then tested for specific binding to aldicarb as compared to other pesticides or herbicides. They were also tested for affinity for aldicarb, using different amounts of culture supernatant as completed by serial concentrations of free aldicarb.

Initial specificity tests with other pesticides or steroids indicated a high degree of specificity for aldicarb, equivalent to that seen from a commercially available sheep polyclonal antisera.

Subclones of these hybridomas were further analysed, yielding at least 2 exhibiting apparent affinity similar to that seen by the sheep antiserum. The most useful clones (those providing the highest signal with the most sensitivity to the competing hapten concentration) were then expanded for antibody production in serum and protein free medium (SIGMA) using a cell factory flask (Integra).

Antibody Purification

Antibodies produced by selected clones were purified by ammonium sulphate precipitation. The precipitates were re-solubilised in PBS, dialysed against three changes of PBS, pH 7.4 and subsequently used for assay development.

Immunoassay Standardisation

Optimal immunoassay conditions were established by means of a checkerboard analysis of the reagents. Immulon-4 ELISA plates were coated with 100μL/well of antibody 124.3 ranging from 0.03 to 5 μg/mL in carbonate buffer pH 9.6 at 4°C overnight. They were blocked with 100 μL/mL of 0.5% w/v BSA (bovine serum albumin) in carbonate buffer, pH 9.6 at room temperature, for 1 h. The plates were then washed three times with TT. 20 μL of TBT was added to the wells followed by a 50 μL aliquot of a 1/5 serial dilution of aldicarb in water. Subsequently a 50 μL aliquot of aldicarb-AP at 1/250 in TBT was added. The reactions were incubated for 4 h at room temperature, and washed three times with TT. 100 μL of *p*-nitrophenyl phosphate substrate in carbonate buffer was added to the wells. The results were quantified with an ELISA plate reader (Parkard Argus 300) at 405 nm.

Subsequent experiments were carried out using the optimal conditions in which Immulon-4 plates were coated using the antibody 124.3 at 1 μg/mL coating buffer and 50 μL of a 1/100 dilution of the aldicarb-AP conjugate. For enhanced sensitivity, the assay was later visualised by the use of Ampli-Q substrate (DAKO). The reactions were quantified on the same reader as above, at 495 nm after a constant developing time.

In attempting to adapt the microtitre ELISA protocol for portable field uses, Nunc Inter Med ELISA tubes were coated with 1 mL of aldicarb-BSA at 10 μg/mL coating buffer overnight at 4°C. The tubes were blocked with 1 mL of 0.5%BSA in coating buffer for 1 h at room temperature. The tubes were washed 3 times with TT. To the tubes, 0.2 mL of TBT was added, followed by 0.5 mL of aldicarb sample in water and finally 0.5 mL of anti-aldicarb antibody, 146.2 at 2 μg/mL in TBT. The mixture was incubated at room temperature for 30 min, washed four times and then 1 mL of ALP-labelled goat anti-mouse IgG Fc antisera was added. After 15 min, the tubes were washed and 1 mL of Ampli-Q was added to each tube. The reaction was stopped

after 15 min with the sulfuric acid stopping solution provided by the manufacturer, and the tubes were read at 495 nm against a water blank on a differential photometer (Millipore).

In a further demonstration of the potential of these MAbs for use at the point-of-need, a prototype lateral flow immunoassay was configured. Essentially, the monoclonal anti-aldicarb antibody 146.2.1 was added to an aliquot of 40 nm gold beads to which polyclonal goat anti-mouse IgG antisera was conjugated. This mixture was exposed to a nitrocellulose membrane on which a line of aldicarb–KLH had been adsorbed. The binding of the gold bead–antibody mixture to this line was assessed in the presence or absence of different concentrations of aldicarb.

RESULTS

Specificity of Monoclonal Antibody 124.3 as Revealed by Hapten Inhibition of Binding to Aldicarb–BSA

In preliminary specificity tests with other pesticides or steroids including the aldicarb metabolites, aldicarb sulfone and sulfoxide, MAb 124.3 exhibited a high degree of specificity for aldicarb, equivalent to that seen from a commercially available sheep polyclonal antiserum (Table I). Antibody 146.2 exhibited similar specificity, with lower affinity. Neither of these antibodies, nor the sheep antisera, reacted with the aldicarb metabolites within the concentration range tested.

Application of Alkaline Phosphatase-labelled Antibody in a Competitive Assay Using Microtitre Plate ELISA

In the process of configuring classic competitive assays in a 96 well ELISA plate format, we found that by immobilising MAb 124.3 on the solid phase to capture analyte, the

TABLE I Specificity of monoclonal antibodies described in this report, as compared with a commercially available sheep polyclonal antisera, as demonstrated by competition for binding to aldicarb-conjugate by various analytes

Pesticides	*Commercial sheep anti-aldicarb antibody (concentration at 50% inhibition)* (ng/mL)	*Mouse monoclonal (concentration at 50% inhibition MAb 124.3)* (ng/mL)	*Anti-aldicarb antibody (concentration at 50% inhibition MAb 146.2.1)* (ng/mL)
Aldicarb	200	200	1000
Aldicarb sulfone	> 1000	> 1000	> 1000
Aldicarb sulfoxide	> 1000	> 1000	> 1000
2,4-dichlorophenoxyl-acetic acid	> 1000	> 1000	> 1000
Atrazine	> 1000	> 1000	> 1000
Alachlor	> 1000	> 1000	> 1000
Carbaryl	> 1000	> 1000	> 1000
Carbofuran	> 1000	> 1000	> 1000
Diflufenican	> 1000	> 1000	> 1000
Metoalachlor	> 1000	> 1000	> 1000
Imazapyr	> 1000	> 1000	> 1000
Oxymal	> 1000	> 1000	> 1000

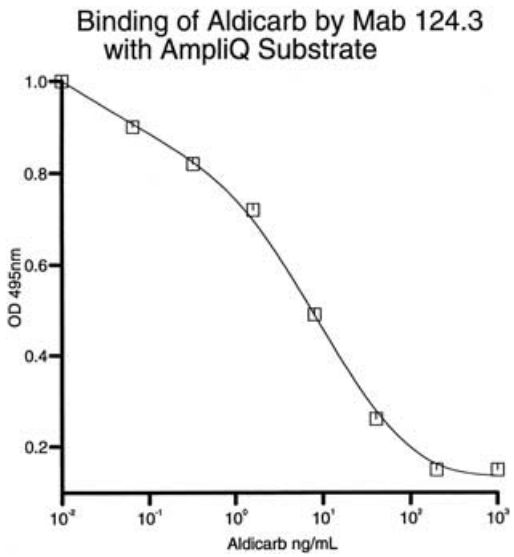

FIGURE 2 Standard curve of aldicarb concentrations competing for the binding of surface-adsorbed antibody 124.3 to aldicarb–ALP in solution.

indirect detection of hapten by the depletion of signal (enzyme-labelled analogue) in unoccupied binding sites could be facilitated with an acceptable lower limit of detection (~1 ng/mL or ppb, just below the inflection point or shoulder of the standard curve), using Ampli-Q substrate (Fig. 2).

Extrapolation from the Microplate Format to Field Conditions

In order to adapt the assay for field use, it was necessary to be able to detect the analyte in only a few minutes – although even shorter reaction times could, in principle, be realised by using a larger volume of analyte. To explore such a possibility, Nunc InterMed ELISA tubes were coated with aldicarb–BSA, and the binding of MAb 146.2.1 was monitored. Figure 3 shows that the lower limit of detection (the region of the standard curve where linearity is curtailed, i.e. the inflection point) was comparable to that obtained in the plate format, with incubation times of only 30 min for the MAb directed to aldicarb, followed by secondary labelled anti-mouse reagents and substrate incubations to provide a read-out within an hour of setting up the assay. On the other hand, in such large format assays, background readings are inevitably higher, and in the experiment shown, complete inhibition of antibody binding at the highest hapten dose (1000 ng/mL) was not demonstrated. Direct labelling of the anti-aldicarb MAb would obviously further decrease the assay time.

The lateral flow format for the immunodiagnostic determination of target analyte was assessed in a hapten-competition approach, in which free aldicarb in water samples competes for the binding of indirect gold-labelled MAb to aldicarb–KLH on nitrocellulose membranes. As shown in Fig. 4, for this preliminary prototype, concentrations of 10 ng/mL can be visualised in minutes by the diminution of signal as compared to samples without any analyte.

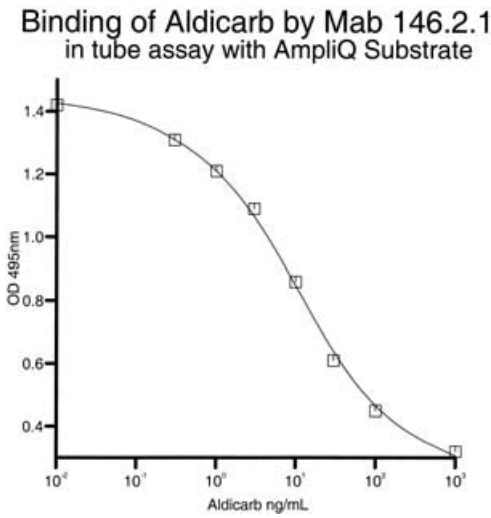

FIGURE 3 Standard curve of aldicarb in 0.5 mL water samples competing with the binding of antibody 146.2.1 to aldicarb–BSA adsorbed to the surface of assay tubes. The assay was developed indirectly using alkaline phosphatase-labelled goat anti-mouse IgG, and Ampli-Q substrate before reading in a hand-held differential photometer.

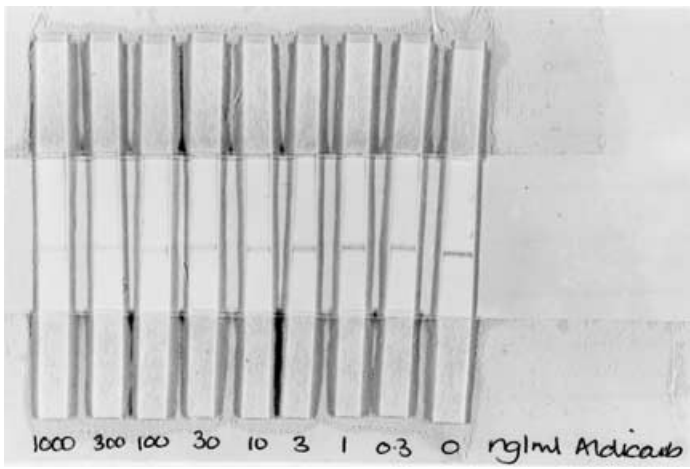

FIGURE 4 Demonstration of aldicarb in water samples by lateral flow immunoassay, where aldicarb competes with antibody 146.2.1 (indirectly gold-labelled using goat anti-mouse IgG conjugated to gold beads) for binding to surface adsorbed aldicarb–KLH on the nitrocellulose membrane.

DISCUSSION

Immunoassays are known to be a low-cost and sensitive way of providing rapid identification of positive samples for further confirmation. However, for suitable robust and reproducible assays to be configured, it is necessary to generate appropriate antibodies with the right affinity and specificity.

Conventionally polyclonal antibodies can be elicited in animal hosts but this approach is always susceptible to batch variability. MAbs, on the other hand, could

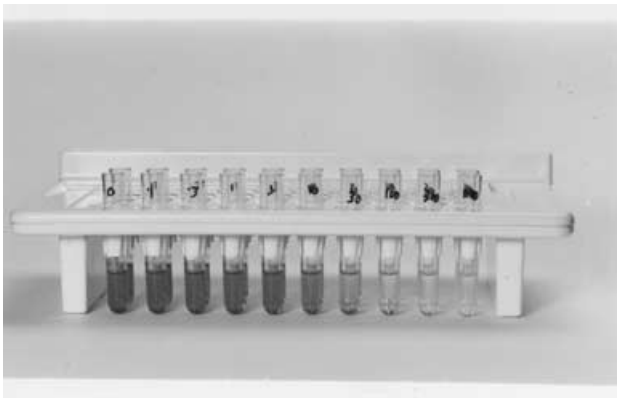

FIGURE 5 A typical tube-based immunoassay for aldicarb, suitable for field conditions.

obviate such a problem, but their generation requires a prolonged labour-intensive procedure. Recognising, however, that polyclonal antisera are currently in use for identifying aldicarb commercially, we considered that a MAb for the specific detection of this important pesticide would constitute a useful advance in the field.

Our initial experiments indicated that these antibodies behaved appropriately in a titratable, acceptably robust manner in the microtitre plate format which is ideal for the diagnostic laboratory.

Accordingly, to explore the full potential of MAbs in terms of assay development, we elected to develop a simple assay format using immobilised antibody on surfaces and to visualise the presence of analyte in water sample by inhibiting the binding of enzyme-labelled or gold-labelled analyte in a competitive manner.

With the development of increasingly effective enzyme substrates for ELISA, sensitivity of assays is enhanced commensurately. Thus, as seen in Fig. 2 with Ampli-Q, the MAb 124.3 can be configured to measure 1 ng of aldicarb per millilitre, in 50 μL samples. Comparable results were also attained in the tube assay, with antibody 146.2.1, catering for field assessment of 0.5 mL samples (Fig. 3). A photograph showing the detectable signal readily visualised with this tube assay is shown in Fig. 5.

Similarly, a prototype lateral flow immunoassay can be demonstrated (Fig. 4), with MAb 146.2.1, using the indirect expedient of gold beads pre-coated with goat anti-mouse IgG, such that 10 ppb (ng/mL) of aldicarb in water samples can be visualised within minutes as a consequence of its preventing the binding of this antibody mixture to aldicarb–KLH adsorbed on nitrocellulose membranes. When optimised, this approach should exhibit even better sensitivities.

It is clear that we have developed a range of immunoassays, using MAbs reactive with aldicarb, which can function either under standard laboratory conditions, or for field use, for the detection of this important pesticide.

Acknowledgements

We wish to thank the Biotechnology Biological Sciences Research Council and the Department of Trade and Industry (UK) for LINK funding which has made this work possible, in addition to support from Rhone-Poulenc Agriculture, now Aventis CropScience, and Selective Antibodies Ltd.

References

[1] R.L. Baron, *Environ. Health Perspect.*, **102** (Suppl. 11), 23–27 (1994).
[2] C. Cox, *J. Pesticide Reform*, **12**, 31–35 (1992).
[3] M.W. Priddle, J.P. Mutch and R.E. Jackson, *Arch. Environ. Contam. Toxicol.*, **22**, 183–189 (1992).
[4] Y. Pico, C. Albelda, J.C. Molto, G. Font and J. Manes, *Food Addit. Contam.*, **7** (Suppl. 1), S29–34 (1990).
[5] S. Chiron and D. Barcelo, *J. Chromatogr.*, **645**, 125–134 (1993).
[6] J.B. Fischer and L.J. Michael, *Bull. Environ. Contam. Toxicol.,* **59**, 611–618 (1997).
[7] C.H. Self, *J. Immunol. Methods*, **76**, 389–393 (1985).
[8] B.F. Erlanger, F. Borek, S.M. Belser and S. Lieberman, *J. Biol. Chem.*, **228**, 713–727 (1957).
[9] E. Harlow and D. Lane, *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, New York (1988).