Comparative Sensitivity of Immunoassays for Haptens Using Monomeric and Dimeric Antibody Fragments

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A single-chain anti-atrazine antibody fragment, scAb (single-chain Fv with a CK domain), was expressed in *Escherichia coli*, and monomeric and dimeric species were preferentially purified from periplasmic extracts by chromatography upon nickel chelate immunosorbent columns or by immunoaffinity purification using a constant domain (CK) tag. Recombinant monomeric and dimeric antibody fragments, Fab, and intact monoclonal antibodies were compared in assays by competition between free atrazine in solution and (a) immobilized atrazine−bovine serum albumin conjugate (indirect assay) or (b) atrazine−alkaline phosphatase (direct assay). Recombinant antibody fragments provided a lower detection limit than either Fab or intact monoclonal antibody in both assay formats. Monomeric fragments displayed a sensitivity of detection down to 0.1 ppb, compared to 1.0 ppb for dimeric fragments and the parental monoclonal.

Keywords: *Antibody fragments; immunoassays; single-chain antibodies; anti-haptens; triazines*

INTRODUCTION

Improvements continue to be made in immunoassay technology using either labeled antigen (competitive approach) or labeled antibody for signal generation. The noncompetitive labeled antibody (immunometric) approach using the two-site "sandwich assay" has led to benefits in diagnostic technology over the competitive assay in terms of speed and sensitivity of assays. Such assays measure the fraction of capture antibody that has bound analyte and, therefore, the intensity of signal increases with increasing amount of analyte in the test sample. By contrast, competition immunoassays measure the "analyte-unbound" sites with the result that low concentrations of analyte are detected by a small reduction in the maximum signal. Such assays therefore generally offer lower limits of detection.

The sandwich assay cannot easily be applied to many small molecular weight analytes such as pollutants, drugs, and metabolites, and major use is currently made of competition assays using labeled antigen. Methods are now being developed to improve the sensitivity of competition assays and bring the advantages of noncompetitive immunoassays to this large group of key chemicals through the use of a variety of labels (e.g., enzymes and fluorescent or chemiluminescent compounds) to replace traditional radioisotope labeling of analyte or antibody, and homogeneous assays that do not require the separation of bound and free moieties before a signal is measured have been devised [for review, Self and Cook (1996)].

Signal generation has been greatly improved using enzyme labels such as alkaline phosphatase and horseradish peroxidase with colorimetric, fluorescent, and chemiluminescent substrates. Biosensor detection methods include enzyme-driven generation of signals for electrochemical detection or direct measurements of antigen-bound antibody with optical, piezoelectric, or electrochemical immunosensors [for review, Gizeli and Lowe (1996)]. One such system involved the immobilization of a derivative of atrazine (a widely used organic herbicide) on the surface of a transparent film and its subsequent exposure to a monoclonal anti-atrazine antibody preincubated with the test sample. The fraction of monoclonal antibody that had not bound atrazine was captured and detected using reflectrometric interference (Brecht et al., 1995). Despite this highly sensitive signal generation system, the detection limit was reported to be 0.25 ppb, which is unlikely to meet the requirements imposed by European Union regulations for a robust assay that can reproducibly detect 0.1 ppb.

Recombinant DNA technology is being exploited in a number of ways to improve immunoassay technology including production in *Escherichia coli* of antibody fragment−enzyme fusion products. In one such system, a fusion product consisting of a dimer of bacterial alkaline phosphatase and fragments of a monoclonal antibody against human IgG was produced in the periplasm of *E. coli* and was effective in an ELISA assay (Ezan et al., 1994). Other systems include genetically engineered lipid-tagged antibody used to construct a fluorescence assay with europium chelate loaded liposomes (Laukkanen et al., 1995) and anti-glycophorin A single-chain antibody fragments (scFv) fused to HIV peptide epitopes to detect antibodies against HIV-1 by agglutination (Coia et al., 1996).

Single-chain antibody fragments (scAbs) are most successfully expressed in *E. coli* by directing nascent polypeptide chains into the periplasm, where the oxidizing environment encourages correct disulfide bond formation [reviewed in Pluckthun (1994)]. ScAb is monomeric, but within the periplasm a proportion spontaneously dimerizes, resulting in a mixture of monomeric and dimeric fragments as illustrated in Figure 1 (McGregor et al., 1994). However, purification

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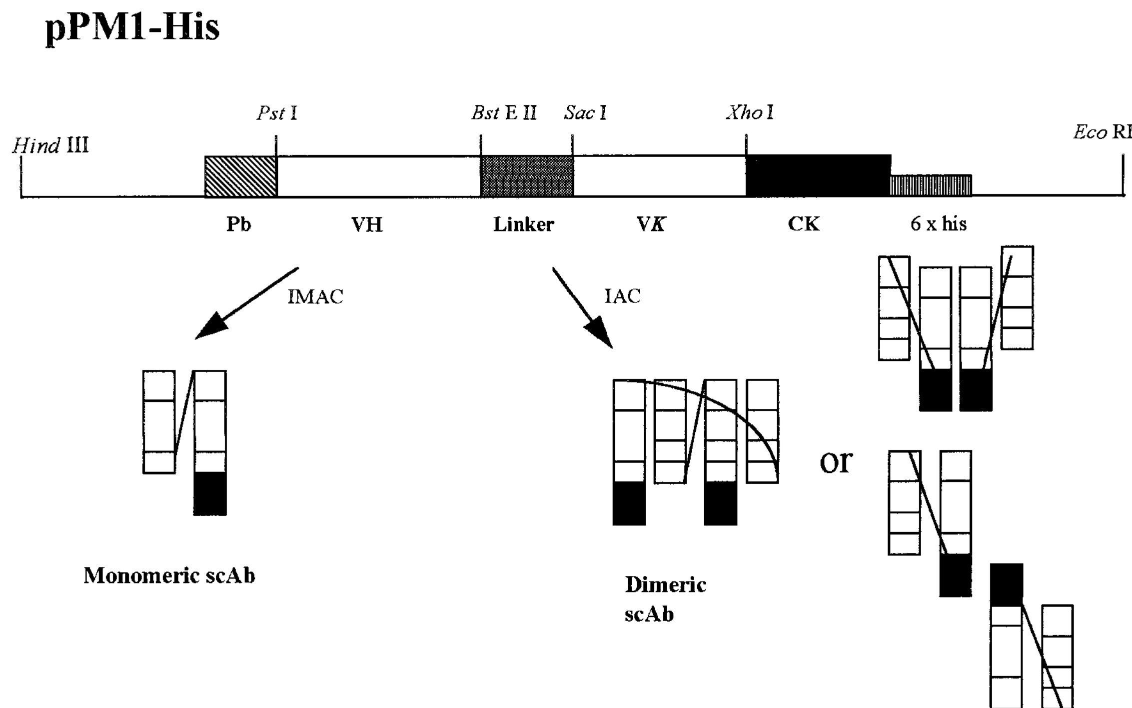

Figure 1. Arrangement of genes in the scAb expression vector pPM1-His. All coding regions were cloned downstream of the *lac* promoter in pUC19. Pb denotes a *Pel* B leader sequence, a human C*K* domain permits detection and purification, and the 6-histidine tail facilitates purification of the fragment. Immunoaffinity purification via the C*K* (IAC) yields predominantly dimeric scAb, whereas purification by IMAC via the 6-histidine tail yields monomeric scAb. Possible structures of dimeric molecules are shown.

protocols can be used to separate the monomeric or dimeric antibody species (Graham et al., 1995; Byrne et al., 1996.)

In this paper we compare the use of purified anti-atrazine monomeric and dimeric antibody fragments with parental monoclonal and enzymatically derived Fab fragments and demonstrate significant increase in the sensitivity of competition immunoassays to detect the organic herbicide hapten atrazine.

The use of monomeric antibody fragments may be particularly useful in providing immunoassays for organic pollutants with the sensitivity required by regulatory authorities.

MATERIALS AND METHODS

Materials. Atrazine–bovine serum albumin (BSA) conjugates were prepared as previously described (Byrne et al., 1996) using the mixed anhydride method to conjugate BSA to the reactive atrazine analogue 2-chloro-4-(iospropylamine)-6-[(carboxypentyl)amino]-*s*-triazine (Dunbar et al., 1990) to provide "Dunbar" conjugate with a C-5 alkyl linker between BSA and atrazine moieties. The hapten load/carrier molecule was determined by matrix-assisted laser desorption mass spectrometry to be between 10 and 12 haptens/BSA molecule. Atrazine–alkaline phosphatase was kindly provided by S. Moyle (Guildhay Ltd. U.K.).

Preparation of Monomeric and Dimeric ScAbs. Histidine-tailed (His) single-chain antibody fragments derived from the anti-atrazine hybridoma 4063-21-1 (Schlaeppi et al., 1989) were obtained from *E. coli XL1*-Blue cells transformed with our vector pPM1-His as previously described (Grant et al., 1995; Byrne et al., 1996). These were grown overnight in 5 mL of Luria–Bertani (LB) medium containing 1% glucose, 12.5 μg/mL tetracycline, and 50 μg/mL ampicillin at 37 °C/250 rpm in a 25 mL universal tube. A 1% final inoculum was used to seed multiple 250 mL baffled flasks holding 50 mL of fresh Terrific broth containing 1% glucose, 12.5 μg/mL tetracycline, and 50 μg/mL ampicillin. The cultures were incubated overnight at 25 °C/250 rpm, by which time the D_{650nm} had reached 12–14. The cells were pelleted by centrifugation at 4000 rpm in a Sorvall RC-5B refrigerated superspeed centrifuge GS-3 rotor at 20 °C for 20 min. The flasks were washed aseptically with sterile PBS, the supernatant was discarded, and the pelleted cells were resuspended in 50 mL of fresh Terrific broth containing 50 μg/mL ampicillin. The cultures were incubated at 25 °C/250 rpm for 1 h prior to induction with a final concentration of 0.1 mM isopropyl β-D-thiogalacoside (IPTG). Expression continued at 25 °C/250 rpm for 3.5–4 h. Cells were then pelleted, the supernatant was discarded, and cells were resuspended in $^{1}/_{25}$th culture volume of ice-cold osmotic solution I (30 mM Tris-HCl, 20% w/v sucrose, pH 8.0, 100 mM EDTA, pH 8.0) added dropwise to a final concentration of 1 mM. Cells were then centrifuged at 10 000 rpm in a Sorvall RC-5B refrigerated superspeed centrifuge SS34 rotor at 4 °C for 20 min, and the supernatant was recovered (T/S fraction). The cell pellet was resuspended in $^{1}/_{25}$th initial culture volume of ice-cold osmotic shock solution II (5 mM $MgSO_4$) and mixed in ice for 5 min. Cells were pelleted as before, and the supernatant ws recovered (M/S fraction). T/S and M/S were filtered through 0.45 μm filters and pooled to provide periplasmic extracts.

Previous studies in our laboratories have shown that crude extracts contain a mixture of monomeric and dimeric scAb (McGregor et al., 1993; Graham et al., 1995; Byrne et al., 1996). Periplasmic extract was purified by either anti-human CK (IAC) or nickel chelate chromatography (IMAC), and purified fragments were analyzed by HPLC analysis using a Hydropore sec83-SO3-C5 size exclusion column (250 × 4.6 mm) eluted with 0.2 M Na_2PO_4 (pH 7.0) at a flow rate of 0.2 mL/min (Byrne et al., 1996). HPLC analysis showed that IMAC-purified material was 90% monomeric, whereas IAC-purified material was 90% dimeric (results not presented). Samples of monomeric and dimeric preparations were held at 4 °C for 1 week and reanalyzed by Biacore analysis to confirm that significant interconversion between monomeric and dimeric forms had not taken place (results not presented).

Purification of Fab. Fab was prepared from 1 mg of mAb 4063-21-1 (Schlaeppi et al., 1989) using Immunopure Fab preparation kit (Pierce, U.K.). Purity was confirmed by show-

ing that the final preparation in the binding assay to Dunbar conjugate gave a strong positive ELISA signal using a Fab-specific antisera and no signal using Fc-specific antisera. A single band of approximate molecular mass 45 kDa was obtained by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) (results not presented).

Biacore Analysis of ScAbs. Real-time biomolecular interaction analysis of the binding of anti-atrazine MAb and scAbs to Dunbar conjugate was performed using a Biacore 2000 instrument (Pharmacia Biosensor, AB). Dunbar conjugate was covalently coupled to the sensor chip flow cells via primary amino groups. This was achieved by passing 35 μL of a 1/800 dilution (~0.04 μg/μL) of Dunbar conjugate in 10 mM sodium acetate, adjusted to pH 4.0, over a suitably treated CM5 chip using the standard Pharmacia Biosensor amine coupling protocol. Kinetic analysis was performed using 1300 resonance units (RU) of bound Dunbar conjugate for the MAbs and 2870 RU of bound Dunbar conjugate for the antibody fragments to generate equivalent signal with different molecular weights. An increase of 1000 RU corresponded to a change in the surface concentration on the sensor chip flow cell of ~1 ng/mm^2 for proteins.

Individual samples consisting of 100 μL of either purified MAb or antibody fragment were passed over the chip surface at a flow rate of 10 μL/min. Binding of the MAbs or antibody fragments to the Dunbar coated sensor chip was displayed as a sensorgram, which plotted changes in the resonance signal as a function of time. As a control, samples were also passed over a flow cell containing BSA coupled to the chip surface. The sensorgram obtained from the BSA flow cell was used to normalize the sensorgram obtained from MAb or antibody fragment binding to the Dunbar conjugate. Following analysis, MAb or antibody fragment bound to the sensor chip surface was eluted using 100 mM HCl, until the resonance signal returned to its initial value. The concentrations of MAb and antibody fragment used were as follows: 4063-21-1 MAb, 2000, 1000, 500, 250, 200, 125, 62.5, 31.2, 15.6, and 7.8 nM; 4063-21-1 CK-purified scAb, 450, 420, 380, 340, 300, 260, 220, 200, 180, 150, 100, and 50 nM; 4063-21-1 IMAC-purified scAb, 450, 400, 350, 300, 250, 200, 150, and 100 nM.

At each concentration, reference surface readings were subtracted and data corrected for baseline and then analyzed using BIA evaluation 2.1, which derives kinetic parameters through nonlinear curve fitting. Good fit was confirmed by residual plot analysis being within ±2 RU. For all data, several kinetic models were compared, and the association model A + B = AB and the dissociation model AB = A + B gave the best fit. Apparent k_d constants were derived as those that were consistent over the analyte concentration ranges devoid of mass action and diffusion limitations. Plots of association constants k_s ($k_s = kC + k_d$) against analyte concentration were linear within this range, and k_a was derived as the slope of this line ($K_A = k_d/k_a$).

ELISA Assays of ScAbs. *(i) Indirect Competition Assays.* Ninety-six-well flat-bottom Immulon 4 ELISA microtiter plates (Dynatech Laboratories Ltd.) were coated with 100 μL per well of atrazine−BSA conjugate (Dunbar conjugate) diluted 1:2000 in PBS, resulting in ~2 ng of conjugate per well. Plates were incubated for 1 h at room temperature and then washed three times with phosphate-buffered saline containing 0.05% Tween 20 (PBST). Plates were blocked with 200 μL of 1% BSA in PBS for 1 h at 4 °C and then washed three times with PBST. A decreasing range of different triazine concentrations (atrazine, propazine, or simazine) was premixed with 50 μL of purified mAb or scAb and incubated for 1 h at 4 °C before addition to the ELISA plate and incubated for a further 1 h at 4 °C. Plates were washed three times with PBST, and then 100 μL per well of goat anti-mouse IgG (Fc specific) peroxidase conjugate diluted 1:1000 in PBS (to detect MAb) or goat anti-human IgG (Fab specific) peroxidase conjugate to detect scAb was added and plates were incubated for 1 h at 4 °C. Plates were washed four times with PBST, the ELISA was developed using 100 μL of 3,3′,5,5′- tetramethylbenzidine dihydrocholoride (TMB), the reaction was stopped by adding 50 μL of 1 M H_2SO_4, and absorbance was measured at 450/405 nm.

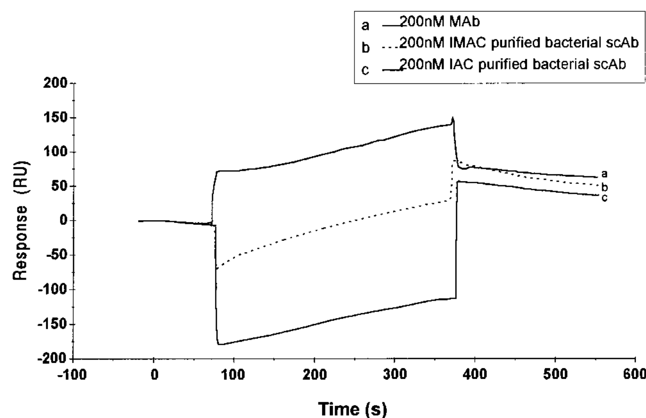

Figure 2. Sensorgram showing association (~75−375 s) and dissociation (~375−550 s) of 200 nM mAb, 200 nM IMAC-purified scAb, and 200 nm IAC-purified scAb applied to a Dunbar conjugated coated sensor chip.

(ii) Direct Competition Assay. Ninety-six-well flat-bottom Immulon 4 ELISA plates (Dynatech Laboratories Ltd.) were coated with 100 μL per well of goat anti-mouse IgG (whole molecule) diluted 1:1000 in PBS (to capture MAb) or goat anti-human IgG K-light chains (bound and free) (to capture scAb) for 1 h at room temperature. Plates were washed three times with PBST and blocked with 200 μL of 1% BSA in PBS. After 1 h at 4 °C, they were washed three times with PBST. Duplicate 50 μL samples consisting of a range of decreasing concentrations of 25 μL of triazine concentrations (atrazine, simazine, or propazine) in H_2O, 25 μL of a 1:1250 dilution of atrazine−alkaline phosphatase tracer in PBS, and 50 μL of purified MAb or ScAb were mixed and incubated at 4 °C for 1 h and then added to the ELISA plates. Plates were incubated at 4 °C for 1 h and then washed four times with PBST. The ELISA was developed by adding 100 μL per well of Blue Phos microwell phosphatase substrate system (2-C) (Kirkegaard and Perry Laboratories, MD); absorbance was measured at 620 nm.

RESULTS

Expression and Purification of Monomeric and Dimeric Antibody Fragments. The heavy and light chain genes from hybridoma cell line 4063-21-1 expressing murine anti-atrazine monoclonal antibody were cloned into the *E. coli* foreign gene expression vector construct pPM1-His as described previously (Byrne et al., 1996). The resulting protein product, referred to as scAb, consists of a single-chain Fv anti-atrazine antibody fragment with a human CK motif and a polyhistidine tail fused to the murine VK and is secreted into the periplasm of *E. coli* on induction of gene expression with IPTG (Molloy et al., 1995). In agreement with previous results (Byrne et al., 1996) scAb purified by nickel chelate chromatography (NTA), which preferentially binds the polyhistidine tail, was 90% monomeric, whereas that purified by anti-CK was 90% dimeric (results not presented). These separate preparations are referred to as monomeric and dimeric scAbs, respectively (Figure 1).

Analysis of Kinetics of Binding of Anti-Atrazine ScAbs. To ensure that as far as possible equal amounts of immunoreactive material were being compared, in preliminary experiments, each sample was immobilized by the CK domain and titrated for binding of atrazine−alkaline phosphatase tracer. Whereas at very low concentrations dimeric fragments showed higher antigen binding, such differences were minimal at concentrations >2 nM (results not presented). Typical sensorgram data are presented in Figure 2 and show the real-

Table 1. Biacore Analysis of Anti-Atrazine mAb and Monomeric and Dimeric scAbs[a]

	k_a (M^{-1} s^{-1})	k_d (s^{-1})	K_A (M^{-1})
mAb	4.83×10^3	3.49×10^{-4}	1.38×10^7
IAC scAb	4.89×10^3	3.64×10^{-4}	1.34×10^7
IMAC scAb	5.57×10^3	2.16×10^{-3}	2.58×10^6

[a] Association constants (k_a), dissociation constants (k_d), and the affinity constants (K_A) for the anti-atrazine mAb and related scAbs were based upon multiple runs at 10 different concentrations.

time rate of association and dissociation of MAb and IMAC- and IAC-purified scAbs from atrazine–BSA immobilized upon the sensor chip. Although the rates of association are similar for all three preparations, IMAC-purified monomeric fragments show significantly more rapid dissociation (Table 1). RU are a direct measurement of the change in concentration of solute in the surface layer resulting from interaction of antigen with antibody. IMAC-purified monomeric fragments provide a calculated dissociation rate of 2.16×10^{-3} s^{-1}, 10-fold faster than dimeric forms. The association rate of monomeric material also appears to be slightly faster, resulting in an overall 5–6-fold reduction in K_A (Table 1). This is probably due to the avidity effect of dimers possessing two antigen binding sites per molecule which can bind the conjugate via either or both antigen binding sites, whereas the monomer can only bind the conjugate with a single antigen binding site. The rapid dissociation of monomeric scFv compared with monoclonal and Fab has been observed by other groups (Abraham et al., 1995; Borrebaeck et al., 1993; Iliades et al., 1997).

Monomeric and dimeric preparations were held at 4 °C for 1 week and reanalyzed. No differences were noted, implying that little change in monomer/dimer equilibrium had taken place with this antibody.

Anti-Atrazine Competition ELISA Assays Using Antibody Fragments. *(i) Indirect Competition Assay.* Preliminary studies demonstrated that concentrations of mAb between 0.5 and 2 nM were subsaturating with a reduction in signal dependent on the concentration of mAb. Though some variation in absolute inhibitor values was evident in different experiments, typical results in Figure 3 indicate that IC_{50} values of the order of 10 nM for mAb, 7.0 nM for dimeric scAb, and 0.7 nM for monomeric scAb are reproducible trends (Grant, 1997), demonstrating the superior sensitivity of monomeric scAb in this assay. Monomeric scAb offers the greater sensitivity in that reduction in signal is detectable with <0.5 nM atrazine. Assays using the atrazine analogue propazine showed the same IC_{50} values as for atrazine, whereas assay with simazine showed a 7–10-fold increase of the corresponding IC_{50} for atrazine (results not presented). The scAbs therefore retain the same specificity with respect to relative binding to the atrazine chemical analogues simazine and propazine, as the parent monoclonal antibody (Schlaeppi et al., 1989).

(ii) Direct Competition Assay. The results in Figure 4 compare the relative sensitivities in a liquid–liquid competition assay from which it can be seen that the IC_{50} at the concentration of tracer used is 25 nM for mAb, 20 nM for Fab, and 3–5 nM for dimeric and monomeric scAb, again demonstrating the superior sensitivity of scAb in this assay.

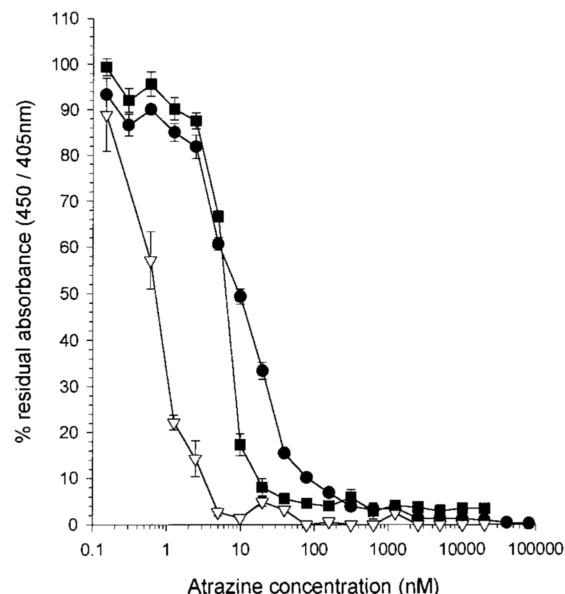

Figure 3. Indirect competition ELISA assay between (●) 2 nM mAb, (■) 20 nM IAC-purified scAb, and (▽) 20 nM IMAC-purified scAb for binding between free atrazine and 1:2000 dilution of immobilized Dunbar conjugate.

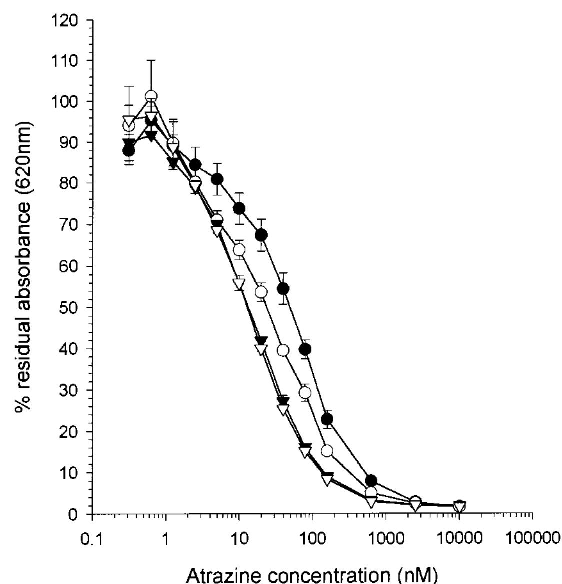

Figure 4. Direct competition ELISA comparing sensitivities to a range of free atrazine concentrations and 1:1000 atrazine–alkaline phosphatase tracer of 1 nM (●) mAb, (○) proteolytically prepared Fab, (▽) Ck purified dimeric scAb, and (▼) NTA purified monomeric scAb.

DISCUSSION

Previous work from our laboratories has demonstrated the cloning and expression of anti-paraquat (Graham et al., 1995) and anti-atrazine (Byrne et al., 1996) antibody fragments in *E. coli.* For the anti-atrazine antibody we have shown that although monomeric and dimeric fragments have reduced binding capacity, compared to mAb, for absorption to atrazine conjugates immobilized upon multiwell plates, the fragments had improved sensitivity of detection in an indirect competition assay.

In this paper we demonstrate that the reduced binding capacity of monomeric scAb is attributable to a faster dissociation rate (Table 1). The scAbs are more sensitive to triazines than either the parental mAb or

Fab in both competition ELISA formats, with monomeric scAb showing a significantly greater signal reduction than dimeric scAb at low concentrations of analyte.

In the indirect competition assay, preincubation of dimeric fragments with a low concentration of atrazine will result in a population of scAbs with only one binding site occupied, leaving the other antigen binding site still available for capture, whereas antigen-bound monomeric fragments will not be captured. This is reflected in Figure 3, where a 20% reduction in signal for monomeric scAb is noted at <0.5 nM atrazine compared to 5 nM for dimers. This difference is not expected with the direct competition assay (Figure 4) as antibody fragments are competing for binding of free atrazine in solution with atrazine−alkaline phosphatase conjugate also in solution, and both monomeric and dimeric species are captured equally.

Reasons for anti-atrazine recombinant fragments showing greater sensitivity in competition assays than monoclonal or Fab fragments are more difficult to provide. The kinetics of binding of monomers and dimers to immobilized hapten are equivalent whether recombinant or not (Table 1). Some differences may be attributable to different capture antisera or residual differences in immunoreactivity, despite the precautions taken in this study, but they are unlikely to explain 10-fold differences. Recombinant single-chain antibody molecules in solution establish a monomer/dimer equilibrium (Whitlow et al., 1993: Desplancq et al., 1994) and may therefore be more flexible and could offer small haptens, such as atrazine when free in solution, easier access to their antigen binding cavities.

A number of anti-*s*-triazine rabbit and sheep polyclonal sera and murine monoclonals have been used successfully in ELISAs to determine atrazine levels in water samples (Schneider et al., 1992: Wittmann and Hock, 1991, 1993; Wust and Hock, 1992: Muldoon et al., 1993: Wortberg et al., 1996) with detection limits of 0.01 μg/L ($IC_{50} = 0.2$ μg/L) to 0.03 μg/L. Recombinant anti-atrazine Fab and scFv antibody fragments have been previously prepared (Ward et al., 1993; Kramer and Hock, 1996), and both showed a 10-fold reduction in sensitivity. In the former case a mutation possibly introduced during cloning may have contributed to the loss of affinity, and in the latter case unpurified cell samples of scFv were used and would contain monomeric, dimeric, and incorrectly folded scFv species, which would distort titration values. Although this paper represents the first observation of improved sensitivity with monomeric scAbs, we have recently confirmed this with an anti-mecoprop scAb (G. Strachan, unpublished results). Bacteriophage display technologies are now being applied to derive anti-atrazine scFvs (Kramer, 1998; Hayhurst et al., 1998) and may provide novel scAbs with improved affinity.

Though absolute sensitivities cannot be directly compared, in the current assay format, monomeric scAbs are of the same order of sensitivity as polyclonal sera. The IC_{50} value of 0.7 nM for monovalent scAb, equivalent to 0.15 ppb of atrazine, with relative standard deviation of 6% (Figure 3), may meet the assay requirements of European legislation levels of 0.1 ppb (Agg Ba and Zabel, 1990), although further studies using environmental samples are required. Monomeric fragments may offer the opportunity for greatly increased sensitivity through their incorporation into other immunoassay detection methods and formats.

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