Production and Characterization of Murine Single Chain Fv Antibodies to Aflatoxin B_1 Derived From a Pre-immunized Antibody Phage Display Library System

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The production and characterization of recombinant antibodies to aflatoxin B_1 (AFB_1), a potent mycotoxin and carcinogen is described. The antibody fragments produced were then applied for use in a surface plasmon resonance-based biosensor (BIAcore), which measures biomolecular interactions in 'real-time'. Single chain Fv (scFv) antibodies were generated to aflatoxin B_1 from an established phage display system, which incorporated a range of different plasmids for efficient scFv expression. The scFv's were used in the development of a competitive ELISA, and also for the development of surface plasmon resonance (SPR)-based inhibition immunoassays. They were found to be suitable for the detection of AFB_1, in this format, with the assays being sensitive and reproducible.

Keywords: *Aflatoxin B_1, single chain Fv, phage display technology, surface plasmon resonance*

INTRODUCTION

Aflatoxins were discovered in 1960 following the deaths of several thousand turkey poults throughout England, due to consumption of contaminated Brazilian groundnut meal (Murray *et al.*, 1982). They are a group of highly toxic fungal secondary metabolites that occur in *Aspergillus* species (O'Kennedy & Thornes, 1997). The fungus contaminates foodstuffs and feeds, as well as crops such as maize, cottonseed, peanuts, and tree nuts during growth but particularly while in storage. Contamination is most common in tropical and sub-tropical countries where humidity is high, and, therefore, allows favourable conditions for the fungus to grow. Aflatoxins are members of the coumarin family and the most significantly occurring compound is aflatoxin B_1 (AFB_1), which is produced by certain strains of *Aspergillus flavus*

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and *Aspergillus parasiticus.* Other aflatoxins designated B_2, G_1, and G_2 are also produced, but AFB_1 is generally present in the largest quantity, and is the most toxic. Aflatoxin M_1 (AFM_1-or 4-hydroxy-AFB_1) is a hydroxylated metabolite of AFB_1, which is excreted in the milk of dairy cattle after they consume aflatoxin-contaminated food. AFB_1 is a very potent carcinogen and has been linked to human hepatocellular carcinoma. The international agency for research on cancer regards it as a human carcinogen (Ward *et al.*, 1990).

Due to the widespread occurrence of the toxin-producing fungi in cereals, major efforts have been made to develop rapid and sensitive methods for detection of aflatoxins. Thin-layer chromatography and high-pressure liquid chromatography have been traditional methods used (Nawaz *et al.*, 1995), but can lack sensitivity and consistency due to the presence of many components in food and biological matrices that can interfere with physiochemical analysis. Immunoassays can offer high levels of sensitivity and specificity, and are capable of high sample throughput (Linskens *et al.*, 1992). Due to the low molecular weight of aflatoxin B_1 (i.e. less than 1000 Da) it must be covalently linked to an immunogenic carrier molecule such as a protein (e.g. bovine serum albumin; BSA), which will elicit a strong immune response following immunization. The protein conjugate is also used at the screening and characterization stages of antibody production.

Antibody engineering has allowed for the construction of large antibody libraries that can be used for the *in vitro* selection of many different molecules. In recent years, phage-display technology has also become increasingly powerful, and has transformed the way in which we produce binding-ligands, such as antibodies or peptides for a given target. In order to produce a recombinant antibody phage display library, mice were immunized with AFB_1-BSA conjugate, and the RNA from their spleens was used for the construction of an antibody phage-display library, which was used to produce soluble scFv antibodies specific for AFB_1. One of the main advantages of phage-display technology is the direct link of DNA sequence to protein function. As a result, single clones can be rapidly screened for antigen binding, as well as being selected from pools in the same experimental set-up. A new and re-designed phage display system has been developed by Krebber *et al.* (1997), which has a number of significantly improved features that include an extended primer mix for amplification of the variable heavy and light chains, directional cloning of the scFv fragment using the restriction enzyme SfiI, as well as a number of vectors that allow for expression of the scFv in various forms.

The genetic material (mRNA) for use in an antibody phage display library can originate from an immunized mouse if one were to produce a pre-immunized phage display library, or alternatively a naïve mouse for production of a naïve library. It is also possible to obtain the genetic material from a hybridoma, and clone the scFv fragment of the parent monoclonal antibody (MAb). The mRNA is then reverse-transcribed to produce DNA. The V_H and V_L fragments are amplified by PCR using the designated primers and the DNA. In the Krebber-based system, splice by overlap extension (SOE)-PCR is used to anneal the V_H-V_L fragments together as one. Restriction enzyme digestion is carried out on the SOE product as well as the vector (pAK 100), which enables ligation of the scFv fragment into pAK 100. This is transformed into *E. coli*, and results in an antibody phage display library. Once the bacteria are harbouring the phagemid vector, they can be superinfected with helper phage to drive production of phage particles carrying the scFv fragment, as a fusion product with the phage coat protein pIII on the surface and genes encoding the scFv inside the particle. Expression of the fusion product and its subsequent incorporation into the mature phage coat results in the ligand being presented on the phage surface, while its genetic material resides within the phage particle. This connection between ligand genotype and phenotype allows the enrichment of specific phage using selection on an immobilized target.

The single chain Fv antibodies produced, were applied in a biosensor system called biospecific interaction analysis (BIA). This is able to measure biospecific interactions (e.g. antigen–antibody binding) in 'real-time'. A commercially available instrument (BIAcore) works on the principle of surface plasmon resonance (SPR) (Quinn & O'Kennedy, 1999). It

detects changes in the refractive index continuously of an antibody and free toxin solution close to the surface of the sensor chip. Aflatoxin conjugate is covalently immobilized onto the surface of the sensor chip and scFv antibody and free toxin is allowed to flow continuously over the surface. The conjugate and free toxin in solution compete for binding to the scFv antibody in solution. As the scFv binds to the conjugate, the refractive index of the buffer in contact with the sensor chip changes. The change in refractive index is measured by SPR. Continuous monitoring of the resonance angle gives a change in the refractive index of the buffer solution close to the metal film surface. This change is then detected and quantified (as response units) by the instrument as a sensorgram. Approximately 1000 response units (RU) is equivalent to a mass change in the surface concentration of 1 ng mm^{-2} (Stenberg *et al.*, 1991). After the binding interaction occurs, the bound antibody can be removed using chaotrophic reagents, which allow the sensor surface to be used repeatedly. BIAcore has been used for applications such as kinetic analysis (Marlborg & Borrebaeck, 1995), inhibition immunoassays for the detection of small molecules such as drugs and pesticides (Wagner *et al.*, 1995; Alcocer *et al.*, 2000; Dillon *et al.*, 2003), as well as proteomic applications (Krone *et al.*, 1997; Nelson *et al.*, 2000). It has also been previously been used in the detection of mycotoxins (Van der Gaag *et al.*, 1998), where aflatoxin B_1 was directly immobilized onto the dextran gel surface for use in an inhibitive immunoassay. It was found that the assay was sensitive enough for detection of aflatoxins in food and feed. SPR-based immunoassays have also been developed for other mycotoxins, such as fumonisin B_1 (Mullett *et al.*, 1998).

The research described in this paper focuses on the exploitation of phage display technology for the production of murine single chain Fv antibodies to aflatoxin B_1 using the 'Krebber'-based system (Krebber *et al.*, 1997). Soluble single chain Fv antibodies were isolated from the library, were characterized and subsequently used for the quantification of free aflatoxin, using an inhibitive indirect sensing method on BIAcore.

MATERIALS AND EQUIPMENT

Caution. Aflatoxin B_1 is carcinogenic and should be handled with extreme care.

All reagents and chemicals were supplied by Sigma Chemical Co. (Poole, Dorset, UK), unless otherwise stated. Carboxy-methylated dextran was obtained from Fluka Chemicals (Gillingham, Dorset, UK) BIAcore 3000™ and CM5 sensor chip were both supplied by BIAcore AB (Uppsala, Sweden).

Immunization Protocol for BALB/c Mice for the Production of scFv Anti-aflatoxin B_1 Antibodies From Splenomic RNA

A stock 5 mg ml^{-1} solution of aflatoxin B_1-BSA (AFB_1-BSA) conjugate was prepared in phosphate-buffered saline solution (PBS, pH 7.3, 0.15 M-NaCl). This was diluted to a concentration of 50 μg ml^{-1} in PBS. An equal volume of Freund's Complete adjuvant was added to 50 μg ml^{-1} of the conjugate. The mixture was vortexed until it formed an emulsion. 6–10-week-old BALB/c mice were injected sub-cutaneously with 250 μl of the immunogen at several sites. On day 21, the mice were re-immunized intraperitoneally with the same dose of immunogen and Freund's Incomplete adjuvant. On day 28 a blood sample was taken from the tail and analysed, along with intraperitoneal boosts. Subsequent blood sampling was continued until a satisfactory titre of greater than or equal to 1/50 000 was obtained. 3–4 days prior to being sacrificed, the mice were injected with 250 μl of 50 μg ml^{-1} AFB_1-BSA conjugate in PBS. This was administered intravenously via the tail vein.

Production of a Single Chain Fv Antibody Library to Aflatoxin B_1

A pre-immunized scFv phage display library was produced according to the method described by Krebber *et al.* (1997). Total RNA was extracted from spleen cells using Trizol Reagent. This is a 'ready-to-use' reagent for the isolation of total RNA from cells and tissues,

based on the procedure of Chomczynski & Sacchi (1987) 7–10 μg of total RNA was reverse transcribed, using random hexamer primers (Promega) to produce cDNA.

For amplification of V_L and V_H antibody genes from total RNA, PCR reactions were performed in 50 μl volumes, containing 6–8 μg of cDNA, 0.1 nmol of L Back (LB) and L Forward (LF) primer mixes for amplification of V_L genes or 0.1 nmol of HB and HF primer mixes for amplification of V_H genes, 0.4 mM-dNTPs with 1.0 mM-$MgCl_2$ used with the reaction buffer supplied by the manufacturers. PCR purification of both the V_H and V_L genes was performed using the Wizard PCR prep DNA purification kit (Promega) followed by assembly into the scFv format using a 20 amino acid linker by SOE-PCR, with minor modifications.

The SOE-PCR products were again gel purified, SfiI digested and ligated following the manufacturers instructions (Promega) into the pAK100 vector (kindly donated by Dr A. Plückthun, Universität Zürich, Switzerland). This was followed by electroporation into *E. coli* XL1-Blue supercompetent cells (Stratagene).

For expression of phage display antibodies, 50 ml of non-expression medium (NE) (2 × TY containing 1% (v/v) glucose and 25 μg ml^{-1} chloramphenicol) was inoculated with 10^9 cells from the library glycerol stocks and grown in a shaking incubator at 250 rpm at 37°C until the optical density at 550 had reached 0.5 AU. 10^{11} VCSM13 helper phage (Stratagene) and 25 μl of 1 M-isopropyl-β-D-thiogalactopyranoside (IPTG) were added and after incubation at 37° C for 15 min without agitation, the culture was diluted with 100 ml of low expression medium (LE) (2 × TY containing 1% (v/v) glucose, 25 μg ml^{-1} chloramphenicol and 0.5 mM-IPTG). The culture was shaken (250 rpm) for 2 h at 26°C followed by addition of 30 μg ml^{-1} of kanamycin. The culture was then allowed to continue shaking at 26°C for a further 8 h. Phage particle precipitation was then carried out using PEG/NaCl and the pellet resuspended in 2 ml of sterile PBS.

Screening for scFv Phage Display Antibodies From a Pre-immunized Anti-AFB_1 Phage Display Library

Synthesis of AFB_1-dextran conjugate. This procedure was performed according to Langone and Van Vunakis (1976) and involved the production of AFB_1 O-carboxymethyl-hydroxylamine, followed by coupling of the activated aflatoxin molecule to amino-dextran using carbodiimide coupling chemistry according to the method described by Hermanson (1996).

Selection of antigen binders by panning. For selection, an immunotube was coated overnight at 4°C with AFB_1-BSA or AFB_1-dextran (AFB_1-Dex) diluted in PBS (50–100 μg ml^{-1}). The tube was washed five times with PBS containing 0.05% (v/v). Tween 20 (PBST) and five times with PBS. This was then blocked with 4% (w/v) milk in PBS and allowed to incubate for 1 h at 37°C. After this time the tube was again washed, and 1 ml of phagemid particles was added to 3 ml of PBS, which in total contained 2% (w/v) milk. This was allowed to incubate in the immunotube for 2 h at room temperature, gently shaking. After washing (as before) bound phage was eluted from the tube by adding 800 μl 0.1 M-glycine/HCl, pH 2.2, for 10 min. This was then neutralized using 48 μl of 2 M-Tris buffer. Phage were then reinfected into *E. coli.* and grown on agar plates overnight.

Single colonies were selected from these plates and used to inoculate individual wells of a 96-well cell culture plate containing 200 μl 2 × TY containing 1% (v/v) glucose, 25 μg ml^{-1} chloramphenicol and 30 μg ml^{-1} tetracycline. Phage particle production was induced and 75 μl of the supernatant produced from each clone was used for analysis of phage by direct ELISA using an anti-M13 rabbit antibody to detect phage.

Positive individual clones were then inoculated into 5 ml of NE medium followed by inducing phage particle production for analysis by competitive ELISA to characterize the

clones specific for free toxin. This was carried out by coating AFB_1-BSA conjugate onto a microtitre (Nunc) plate, and adding the phage-scFvs to the plate in the presence of free toxin. The bound phage-scFv's were then detected using an anti-M13 rabbit antibody.

Insertion of V_H-V_L Fragment Into pAK 400 Plasmid Vector for Expression of Soluble scFv Antibodies

Plasmids possessing the specific antibody genes were purified and digested with SfiI followed by gel purification of the scFv fragment. This was followed by ligation into previously digested pAK400 vector. Calcium-chloride-competent *E. coli* (JM 83) cells were transformed with pAK400 vector harbouring the plasmid containing the heavy and light chain genes for the scFv to AFB_1.

For soluble expression, 20 to 200 ml expression medium (EM) (2 × TY containing 25 $\mu g\,ml^{-1}$ chloramphenicol) was inoculated with 200 µl to 2 ml of preculture (JM83 harbouring the expression vector for AFB_1), and allowed to grow at 37°C with shaking (250 rpm) until the optical density at 600 nm had reached 0.5 AU. 1 mM-IPTG was added and the culture allowed grow for 4 h at 24°C. The culture was centrifuged at 4000 rpm for 10 min and the supernatant containing soluble scFv antibodies stored at 4°C. A 5 ml ProBond™ Resin (InVitrogen) column was used for the affinity purification of the scFvs. SDS-PAGE and immuno blotting analysis were used to assess antibody purity, as well as confirm the presence of the scFv in samples.

Competitive ELISA Using Soluble scFv Antibodies For Detection of Aflatoxin B_1

Microtitre plates (Nunc Immunoplate Maxisorp, Gibco Ltd., Uxbridge, UK) were coated by adding 100 µl of AFB_1-BSA conjugate dissolved in PBS to each well. The plates were incubated overnight at 4°C. The plates were emptied and washed six times, three times with PBST and three with PBS only. The plate was then blocked by addition of 100 µl per well of PBS containing 2% (w/v) milk powder and incubated for 1 h at 37°C (for both coating and blocking of plates, incubation steps could also be carried out at 37°C for 1 h or 4°C overnight).

The optimal dilution of the scFv antibodies, and the optimal conjugate concentration, for use in competitive ELISA was determined by testing doubling dilutions of scFv against decreasing concentrations of conjugate. This protocol was carried out in the absence of free toxin. From the titre curves obtained, the antibody dilution that gave half the maximum absorbance, and the lowest conjugate concentration that provided sufficiently high absorbances was chosen.

Stock aflatoxin B_1 solution was prepared at a concentration of 2 $mg\,ml^{-1}$ in methanol and diluted in PBS containing 5% (v/v) methanol to produce a set of standard solutions ranging in concentration from 98–1560 $ng\,ml^{-1}$. The plates were washed as before and 50 µl of scFv anti-AFB_1 antibody was added into each well with 50 µl of aflatoxin B_1 standards. The plate was incubated for 1 h at 37°C, followed by washing, and the addition of a 1/400 dilution of anti-FLAG MAb. After incubation and washing, 100 µl of horseradish peroxidase (HRP)-conjugated-anti-mouse secondary antibody, diluted in PBS, was added to each well and incubated for 1 h at 37°C. The plates were emptied and washed. 100 µl of substrate (0.4 $mg\,ml^{-1}$ *o*-phenylenediamine (o-PD), in 0.05 M-phosphate citrate buffer, pH 5.0, and 0.4 $mg\,ml^{-1}$ of urea hydrogen peroxide) was added into each well and incubated for 30 min at 37°C. All absorbance values were measured on a Titertek Twinreader Plus plate reader at an absorbance of 405 nm.

Measurement of Cross-reactions

Stock solutions of aflatoxin B_2, M_1, M_2, G_1, G_2, B_{2a}, and G_{2a} were prepared in methanol and diluted in PBS-5% (v/v) methanol with a range of concentrations from 31.25 to 500 $ng\,ml^{-1}$.

The assay was carried out in the same way as that for the competition ELISA except that standards of the other aflatoxins were added to the plate with the anti-AFB_1 antibody.

Preparation of Grain Matrix Samples

AFB_1 samples in grain matrix were supplied as a gift from Mrs Sue Patel, RHM Technology Ltd, The Lord Rank Centre, London Road, High Wycombe HP12 3QR, UK. A blank sample of grain was extracted according to the method described by Scudamore and Patel (2000), and then spiked with different concentrations of AFB_1 according to the official AOAC methods of analysis (Scott, 1995). The samples were then diluted to a final methanol concentration of 5% (v/v).

Coupling Reaction of Aflatoxin B_1-BSA to cm-Dextran Gel

The carboxymethylated dextran (cm-dextran) matrix was activated by mixing equal volumes of 100 mM-NHS (N-hydroxysuccinimide), and 4 mM-EDC (N-ethyl-N- (dimethyl-amino-propyl) carbodiimide hydrochloride) and injecting the mixture over the sensor chip surface for 7 min at a flowrate of 5 μl min^{-1}. The interactant to be immobilized (concentration-50–200 $\mu g\,ml^{-1}$) was dissolved in 10 mM-acetate buffer (at the required pH), and injected over the surface for 20 min at a flow-rate of 2 μl min^{-1}. The unreacted sites on the sensor chip surface were then capped by injection of 1 M-ethanolamine, pH 8.5, for 7 min.

Sample Preparation for Sensor Analysis

The scFv antibody was diluted in Hepes-buffered saline solution (HBS running buffer, pH 7.4). All buffers and solutions used were made up using ultrapure water, de-gassed and sterile filtered.

Aflatoxin B_1 Preparation for Use in Inhibitive Assay

A 2 $mg\,ml^{-1}$ solution of free aflatoxin B_1 was prepared in methanol. Standards were prepared in PBS containing 5% (v/v) methanol, at concentrations ranging from 0.75 and 195 $ng\,ml^{-1}$. Each sample was incubated with an equal volume of a 1/4 dilution (final dilution-1/8) of anti-aflatoxin scFv antibody for 10 min, and then passed over the surface of the chip. This was carried out three times for each concentration. Regeneration of the surface of the chip was carried out using 10 mM-NaOH.

RESULTS

Estimation of Mouse Titres

Two Balb/C mice were immunized with a commercially available AFB_1-BSA conjugate. The mice received an intra-peritoneal injection of the conjugate together with an equal volume of Freund's Complete adjuvant. Blood was drawn periodically to estimate the specific antibody titre. The final titre for serum from mouse 1 was 1/153 600, while the final titre for serum from mouse 2 was 1/76 800.

Amplification of Mouse Heavy and Light Chains Using PCR

The V_L back primer mix (LB 1–17 and LBλ, representing a total of 131 variants) paired with five V_L forward primers (LF 1, 2, 4, 5 and LFλ) were used to amplify the V_L domains. The V_H back mix (HB 1–19, representing a total of 94 variants) coupled with four V_H forward primers (HF 1–4) were used to amplify the V_H domains. The domains were amplified from a mixture of cDNA obtained from mice 1 and 2, respectively. Using the multiplex PCR protocol, single amplified bands ranging from 375–402 bp for the V_L, and 386–440 bp for V_H were achieved. Figure 1 shows typical bands obtained from the multiplex PCR reaction,

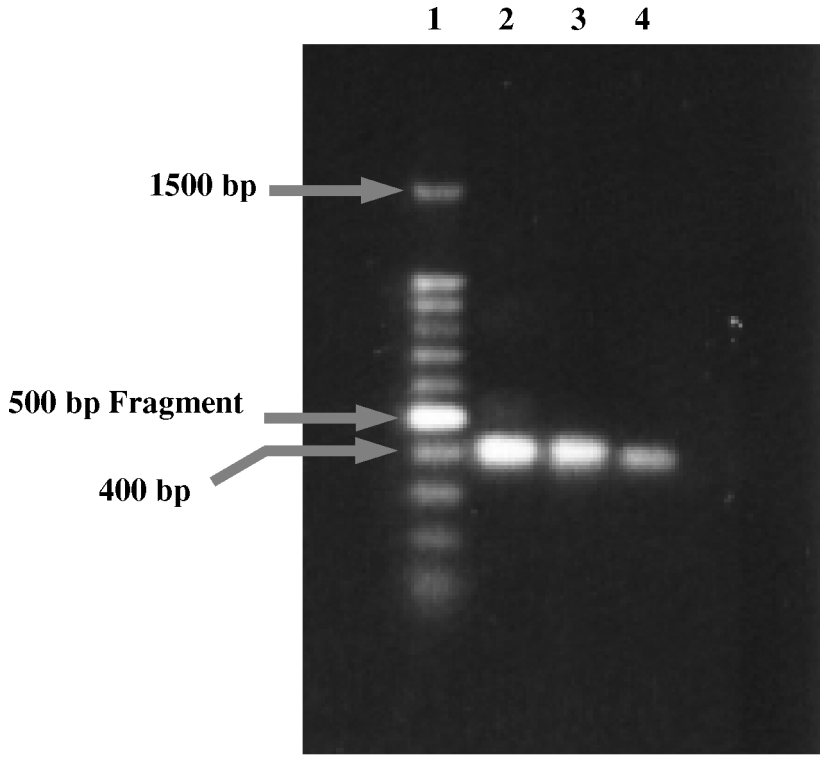

FIG. 1. Amplification of murine heavy (V_H) and light chains (V_L) from a mixture of DNA obtained from two mice immunized with AFB_1-BSA conjugate. Lane 1: 100 base pair (bp) ladder. Lanes 2–3: Amplified heavy chains. Lane 4: Amplified light chains. In the case of the heavy chains, a sharp band with the predicted size 375–402 bp was obtained, while for the light chain, a sharp band with the predicted size 386–440 bp was obtained. These bands were observed as expected just below the 500 bp fragment on the gel picture.

where two batches of heavy chains were amplified, and one batch of light chain was amplified.

Preparation of SOE-PCR Product and pAK 100 for Ligation Reaction

An SOE-PCR was carried out to anneal the heavy and light chains together to form an 800 bp fragment. The product was digested with the restriction enzyme SfiI. Figure 2 shows a gel photograph of the SOE-PCR product as well as the digested PCR product. Only a small change in weight is observed between undigested and digested product. However, there is an apparent difference between the two fragments. This showed the digestion was successful. Figure 3 shows a gel photograph of the restriction enzyme digestion of pAK 100. It is easy to differentiate between the cut vector (at approximately 4144 bp) and the tetracycline gene (2101 bp), which was cut from the vector. The photograph also shows that a small amount of the vector also remained uncut. However, there was sufficient vector digested to gel-purify and use for the ligation reaction.

Production of AFB_1-Dextran Conjugate

An AFB_1-dextran conjugate was synthesized for use in the panning procedure, as it is desirable to use more than one hapten-carrier conjugate when selecting for scFv antibodies

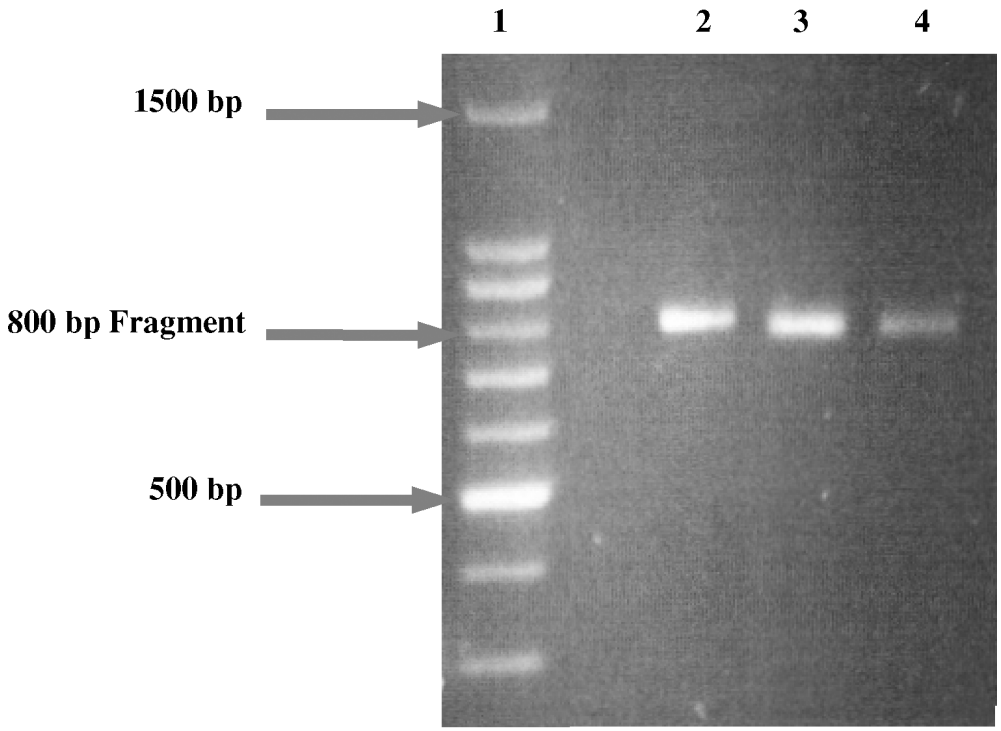

FIG. 2. Restriction enzyme digest of SOE-PCR product using SfiI. Lane 1: 100 bp ladder. Lane 2: Undigested SOE product. Lane 3: Digested PCR product. Lane 4: Digested PCR product. Only a minor change is observed between the digested and undigested bands, as only a small number of base pairs are removed from the DNA fragment, and hence a small change in separation.

to small molecules by phage display. The conjugate was produced, and its performance was assessed using BIAcore analysis (data not shown). This was carried out by passing a polyclonal anti-aflatoxin B_1 antibody preparation over an immobilized AFB_1-dextran surface. Binding of the antibody to the dextran conjugate was observed, indicating that conjugation of AFB_1 to amino-dextran was successful. However, the antibody binding response was low, indicating a low substitution ratio of the hapten to the amino-dextran.

Panning Procedure for the Detection of scFv Phage Display Antibodies

A phage display library consisting of 5×10^3 clones was produced by heat-pulse transforming supercompetent *E. coli* cells with the ligated plasmid DNA. Three rounds of panning were carried out on the library, using an AFB_1-BSA conjugate (rounds 1 and 3), and an AFB_1-dextran conjugate (round 2). After each round of panning, phage titres were carried out in order to assess the progress and efficacy of the panning procedure. 96 random bacterial clones from each round of panning were also selected for analysis, and phage was produced from them. These phage- displaying scFv antibodies were then analysed by phage ELISA for recognition of AFB_1-BSA conjugate. If it was found that the clones recognized the conjugate, they were then analysed for recognition of free toxin. Table 1 shows a summary of the results obtained from the three rounds of panning including phage titres, number of clones assayed after each round, and the number that recognized free toxin.

Eight positive clones recognising AFB_1-BSA conjugate were obtained after three rounds of panning. Seven of the clones were analysed and tested for activity to free aflatoxin B_1.

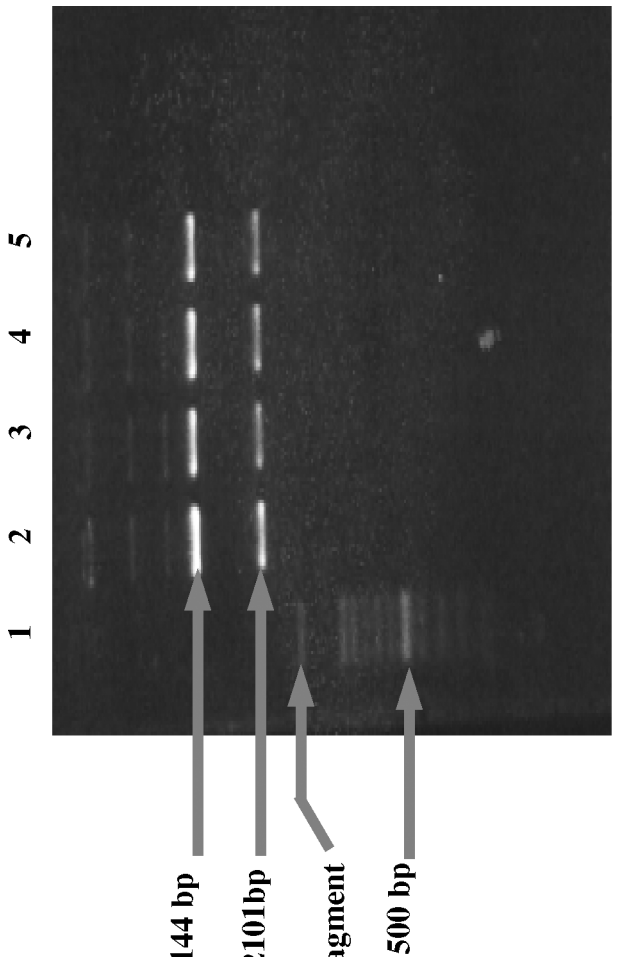

FIG. 3. Restriction enzyme digest of pAK 100 using SfiI. Lane 1: 100 bp ladder. Lanes 2–5: Digested pAK 100. Both the cut tetracycline gene and the digested plasmid can be observed on the gel photo. Three weak-intensity bands are also visible above cut plasmid. These may represent some remaining uncut plasmid as well as partially cut products.

TABLE 1. Data obtained from three panning rounds of an antibody phage-display library produced for the detection of an scFv fragment to aflatoxin B_1. The library was produced from RNA obtained from a mouse that was pre-immunized with the AFB_1-BSA conjugate prior to using the RNA

	Conjugate used for panning	Phage Titre cfu ml^{-1}	No. of clones assayed	No. of clones recognising AFB_1-BSA conjugate	No. of clones recognising free toxin
Phage Production	–	2.5×10^9	96	0	0
Round 1 of panning	AFB_1-BSA 50 $\mu g\,ml^{-1}$	3.0×10^5	96	8	0
Round 2 of panning	AFB_1-Dextran 200 $\mu g\,ml^{-1}$	2.8×10^7	96	10	0
Round 3 of panning	AFB_1-BSA 50 $\mu g\,ml^{-1}$	2.4×10^7	96	7	6

This was carried out using three different concentrations of aflatoxin B_1, 1, 50 and 100 $\mu g\,ml^{-1}$, respectively. Six of the clones showed recognition for free aflatoxin B_1. Figure 4 shows this result. Non-specific interactions were minimal, as the clones did not recognize BSA or milk-coated wells (data not shown).

Production of Soluble scFv Antibodies

Once it was confirmed that the positive clones recognized free AFB_1, three of them were chosen (B3, C4 and F4) for production as soluble scFv antibodies. In order to do this, the

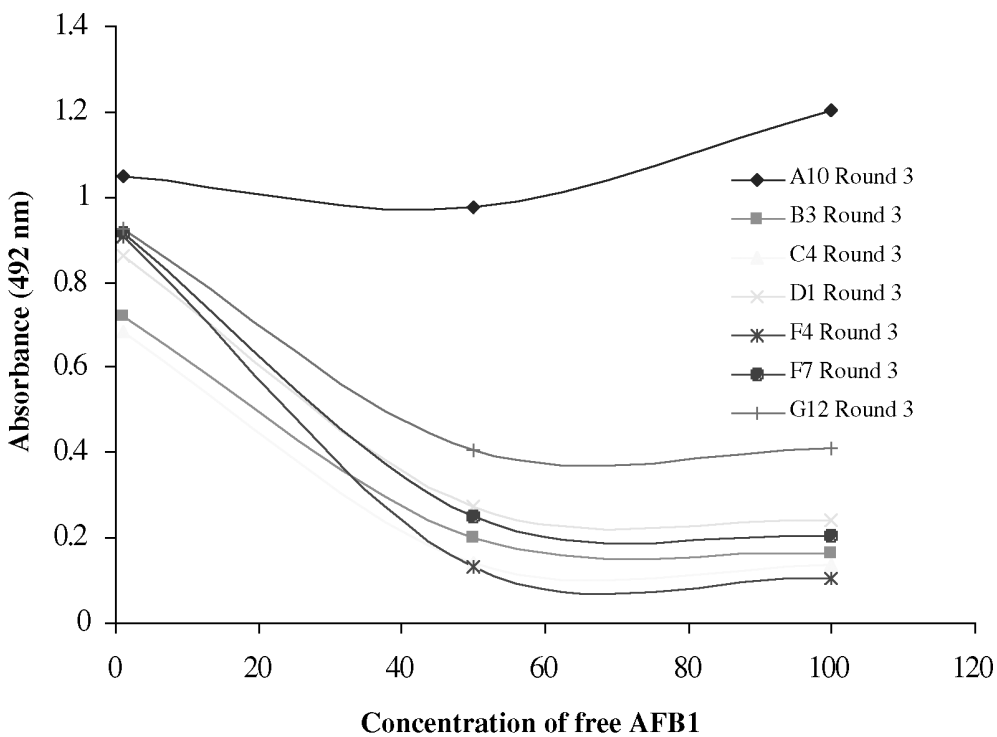

FIG. 4. Competition phage-ELISA of seven clones selected after three rounds of panning for recognition to free AFB_1. All seven of the positives were analysed for recognition to free AFB_1 by phage ELISA. Six of the clones selected showed recognition for the free toxin over the range 1–100 $\mu g\,ml^{-1}$.

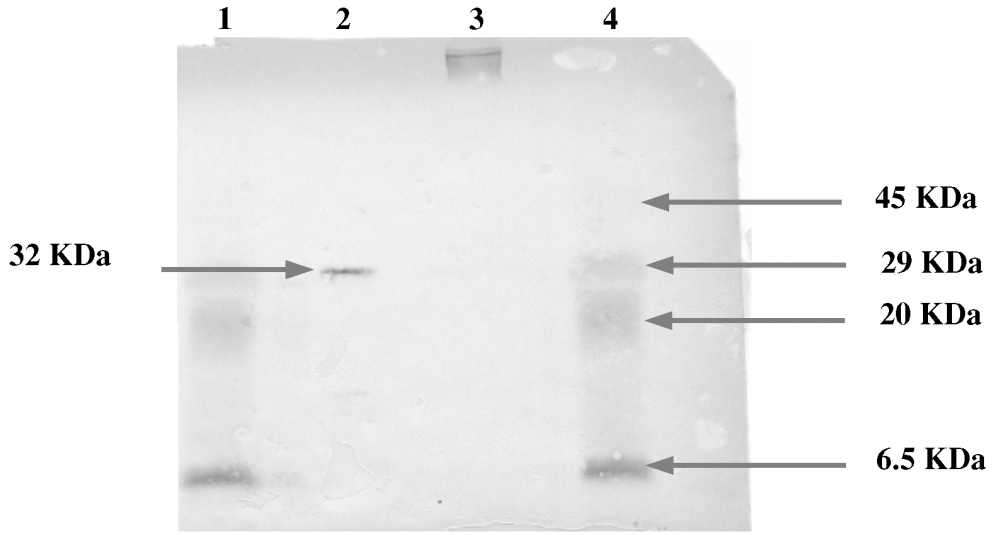

FIG. 5. Immunoblot analysis of IMAC-purification of C4 clone. Lanes 1 and 4: Coloured molecular weight markers. Lane 2: IMAC-purified supernatant. Lane 3: Supernatant. The 32 KDa protein fragment is more apparent in the purified fraction than the supernatant, mainly because the purified fractions were concentrated five-fold after the purification procedure.

bacteria containing the 'positive' plasmid DNA was grown on 5 ml scale and the DNA was purified from the bacteria. The plasmid DNA was restriction enzyme digested using SfiI, and electrophoresed on a low-melt agarose gel. The cut 800 bp fragment separated on the gel, and was purified. The 'positive' scFv DNA was then ligated into pAK 400 and successfully transformed into the non-suppressor *E.coli* strain JM 83. Stocks of the transformed bacteria were made, and used to inoculate media for induction of scFv production.

Soluble scFv expression was induced on the transformed JM 83 cells as described by Krebber *et al.* (1997). Culture supernatants and periplasmic lysates of the three clones were analysed for soluble expression of the scFv fragments by non-competitive ELISA. The culture supernatants and the periplasmic lysates from three clones were analysed (B3, C4 and F4). From this, it was decided to work with only one clone from this stage, and therefore, scFv clone-C4 was chosen for production of soluble scFv antibodies on a large scale and to purify for further analysis. In order to confirm the presence of scFv antibody in the purified samples, an immunoblot was carried out. Detection of the scFv was achieved using an anti-FLAG antibody. Figure 5 shows a picture of the immuno blot. It can be seen that the 32 KDa fragment is quite clear and specific in the purified sample. However, it cannot be seen in the culture supernatant sample, as it may not be concentrated sufficiently.

ELISA Analysis of Soluble scFv Antibodies

A checkerboard ELISA was carried out in order to determine the optimal antibody dilution and conjugate concentration for use in competitive ELISA. From this, it was decided to use a 1/4 dilution of antibody in the competition ELISA, with a conjugate concentration of 12.5 $\mu g\,ml^{-1}$. The competition ELISA produced a linear range of detection for the scFv antibody between 98 and 1560 $ng\,ml^{-1}$ (data not shown), and was found to be reproducible over three days.

Cross-reactivity studies were also carried out on the scFv antibody to seven other structurally related aflatoxins. Standard curves for each of the aflatoxins were produced, and the results were normalized and plotted. The slope of the line of the standard curve for each

TABLE 2. Cross-reactions of scFv anti-AFB_1 antibody (C4) observed to seven other aflatoxins. Standard curves were produced for each of the aflatoxins in the same way as that for AFB_1. Each concentration for each point on the standard curve was assayed in triplicate and the means plotted and normalized in order to construct standard curves

Aflatoxin	scFv % Cross-reactivity
B_1	100
B_2	53
G_1	76
G_2	68
M_1	50
M_2	23
B_{2a}	18
G_{2a}	22

toxin was expressed as a percentage of the slope of the line for binding to AFB_1. The antibody showed a high level of cross-reaction to the structurally related aflatoxins, with the percentage of cross-reactivity for each shown in Table 2.

Background Development of Antibody-based Assay on BIAcore

After confirmation that the scFv antibody recognized free toxin in ELISA, the development of an inhibition surface plasmon resonance-based assay was evaluated. Non-specific binding of the scFv antibody was examined. The AFB_1-BSA conjugate, and BSA were immobilized onto separate sensor surfaces. There was negligible non-specific binding of the scFv to the BSA protein compared to AFB_1-BSA, or to the cm-dextran surface.

Efficiency of Regeneration

The scFv antibody gave an excellent binding response to aflatoxin B_1. A 1/8 dilution of the antibody was found to be sufficient to achieve significant binding to the immobilized conjugate surface. Regeneration studies, using a 1/8 dilution in HBS of scFv anti-AFB_1 antibody showed that it was easily regenerated from the AFB_1-BSA immobilized surface using a 1 min pulse of 10 mM-NaOH. This completely removed all bound scFv antibody after each binding-regeneration pulse. Figure 6 shows a graph of the reproducibility of regeneration for the scFv antibody over 50 regeneration cycles. Binding of the scFv antibody to the conjugate surface was highly reproducible, with approximately 250 RU of antibody binding to the surface each time. The regeneration solution (10 mM-NaOH) did not affect the binding throughout the regeneration study.

BIAcore Assay

An inhibition assay was then developed by immobilising 50 $\mu g\,ml^{-1}$ of aflatoxin B_1-BSA conjugate prepared in 10 mM-sodium acetate, pH 3.9. Standards of free aflatoxin were prepared ranging in concentration from 0 to 195 $ng\,ml^{-1}$. Each sample was incubated with an equal volume of a 1/4 dilution (to ensure a final dilution of 1/8) of the scFv for 1 h a 37°C, and then randomly passed over the surface of the chip for each concentration three times. The binding of antibody to the surface of the chip was inversely proportional to the amount of free aflatoxin in solution. Figure 7 shows the relationship between the number of Response Units bound and the concentration of free aflatoxin B_1. The intra-day variability assay had a range of detection between 3 and 195 $ng\,ml^{-1}$. Inter-day variation was also carried out with the

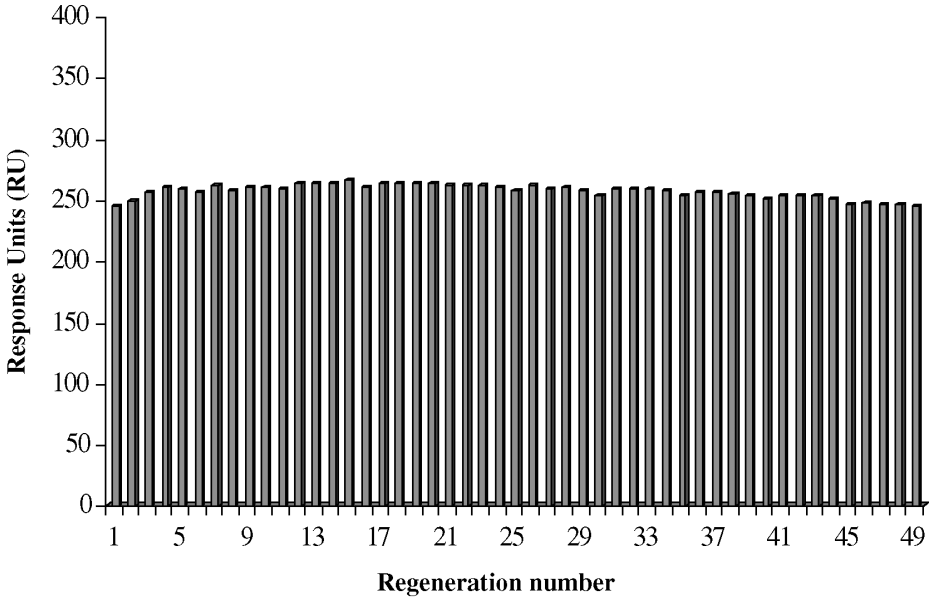

FIG. 6. Graph showing the reproducibility of regeneration of a sensor chip immobilised with AFB_1-BSA on the surface. Fifty consecutive regeneration cycles of a 3 min binding pulse of scFv anti-AFB_1 were carried out. This was followed by a 1 min injection of 10 mM-NaOH as the regeneration solution. This completely removed all of the bound antibody after each binding cycle, which resulted in highly reproducible binding cycles, as no significant decrease in the measured binding response over the course of the regeneration study was observed.

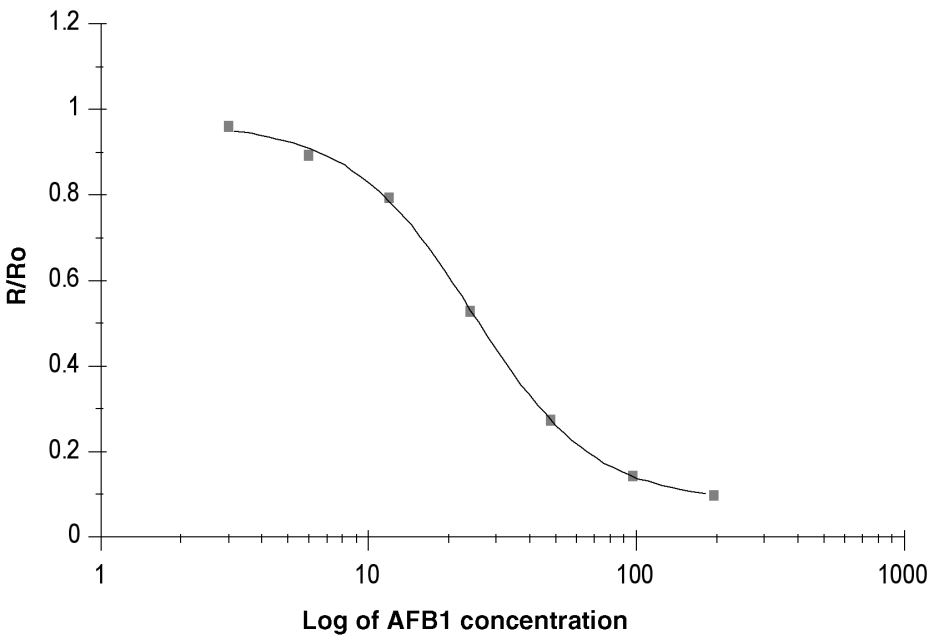

FIG. 7. Inter-day assay curve for AFB_1 in PBS using scFv anti-AFB_1 antibody. The range of detection for the antibody was between 3 and 195 ng ml^{-1}. The calibration plot was constructed using BIAevaluation 3.1 software package. The results shown are the average of triplicate results.

TABLE 3. Inter-day coefficients of variation for scFv anti-AFB_1 antibody. Three sets of eight standards were run on three different days, and the measured binding responses were used to calculate the normalised binding response values. From these the CVs were calculated as the standard deviation expressed as a percentage of the mean normalized values for each standard

Aflatoxin B_1 concentration ($ng\,ml^{-1}$)	Calculated mean ± SD (R/R_0)	Coefficient of variation (%)
195	0.09 ± 0.01	11.06
98	0.14 ± 0.01	12.39
48	0.27 ± 0.05	19.95
24	0.53 ± 0.02	4.01
12	0.79 ± 0.02	3.5
6	0.89 ± 0.03	3.95
3	0.96 ± 0.03	3.35

assay being reproducible over three days between 3 and 195 $ng\,ml^{-1}$. Table 3 shows the coefficients of variation for the inter-day assay.

Detection of AFB_1 in Spiked Grain Samples

Spiked grain samples were prepared as described in materials and methods, and applied in an inhibition assay for the scFv anti-AFB_1 antibody in the same manner as outlined previously.

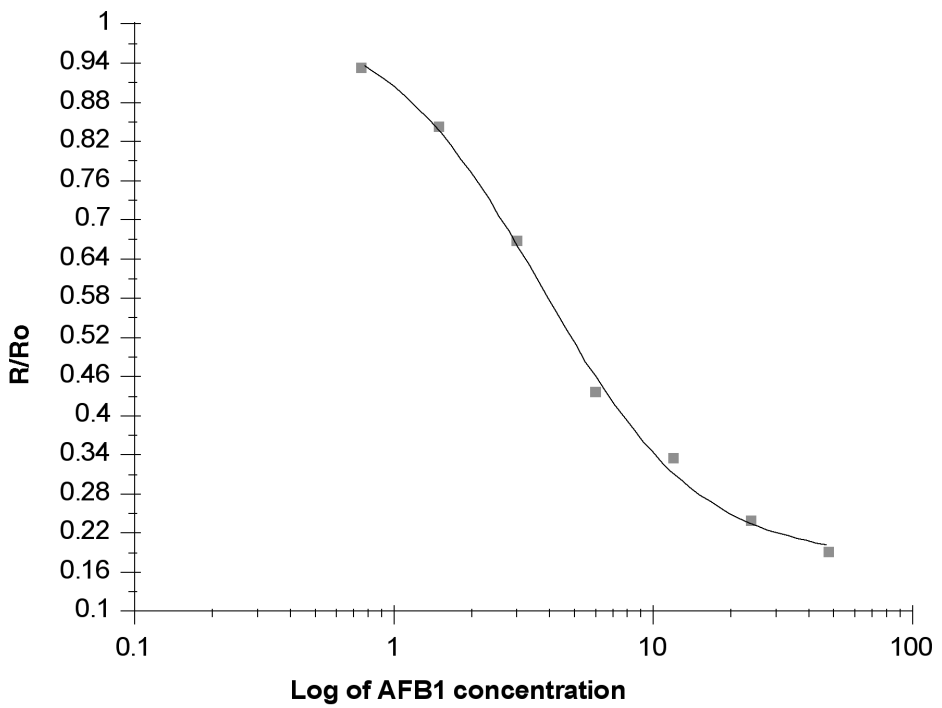

FIG. 8. Inter-day assay curve for AFB_1 in grain samples spiked with AFB_1 using scFv anti-AFB_1 antibody. The range of detection for the antibody was between 0.75 and 48 $ng\,ml^{-1}$. The calibration plot was constructed using BIAevaluation 3.1 software package. The results shown are the average of triplicate results.

TABLE 4. Inter-day coefficients of variation in grain samples spiked with AFB_1 using scFv anti-AFB_1 antibody. Three sets of eight standards were run on three different days, and the measured binding responses were used to calculate the normalised binding response values. From these the CVs were calculated as the standard deviation expressed as a percentage of the mean normalized values for each standard

Aflatoxin B_1 concentration ($ng\,ml^{-1}$)	Calculated mean ± SD (R/R_0)	Coefficient of variation (%)
48.0	0.19 ± 0.007	4.13
24.0	0.24 ± 0.01	7.47
12.0	0.33 ± 0.04	11.83
6.0	0.44 ± 0.01	4.06
3.0	0.67 ± 0.02	3.58
1.5	0.84 ± 0.03	4.63
0.75	0.93 ± 0.03	3.75

Regeneration was also carried out using 10 mM-NaOH. The intra-day assay curve for the range detection for the scFv antibody was between 0.75 and 48 $ng\,ml^{-1}$, with percentage CVs between 0.1 and 16.5%. Inter-day variation studies were also carried out by running the assay on three separate days, normalising the results, and calculating the percentage CVs between the assays. Figure 8 shows the inter-day assay curve with a range of detection for the scFv antibody between 0.75 and 48 $ng\,ml^{-1}$. Table 4 shows the percentage CVs which varied between 3.7 and 11.8%.

DISCUSSION

Balb/c mice were immunized with an AFB_1-BSA conjugate, and once a sufficiently high enough specific antibody titre was obtained, the spleen was removed from the mice, and the RNA extracted from it.

Amplification of the heavy and light chains was carried out using the extended primer mix, which incorporated all of the mouse V_H, V_λ, and V_κ sequences collected in the Kabat data base (Kabat *et al.*, 1991). It also combined the extended primer sets described by Kettleborough *et al.* (1993), Ørum *et al.* (1993), and Zhou *et al.* (1994). This ensured that a larger repertoire of DNA coding for the mouse heavy and light chains was effectively and specifically amplified, and as a result a larger population of antibodies available for selection during the screening process. It can be seen (Figure 1) that the heavy and light chains were specifically amplified, using an annealing temperature of 63°C. Approximately 10 ng of each of the V_H and V_L was used for the SOE-PCR. This produced an 800 bp fragment, as the heavy and light chains are approximately 400 bp long each.

Both the SOE-PCR product and the vector pAK 100 had to be restriction enzyme- digested prior to the ligation step. SfiI was the restriction enzyme used, as it cuts two sites at once on a piece of DNA. It is also useful, as the sites at which it cuts at are quite rare in antibody sequences, and therefore, it eliminates the possibility of internal digestion, and incorrect sequence lengths. Digestion of the SOE product results in a very small change in weight of the piece of DNA. It is therefore quite difficult to differentiate between cut and uncut DNA. In order to aid the visual differentiation, the samples were electrophoresed on a 2–2.5% agarose gel, which improved the separation of the fragments. Figure 2 shows an agarose gel of digested and undigested fragment, and it is just possible to differentiate between the two fragments on the gel, indicating the reaction was successful. Digestion of the pAK 100 vector was visually easier to differentiate, as the cut tetracycline cassette is 2101 bp in length. This was easily visualized on the gel (Figure 3). It can also be seen on the gel that a certain amount

of vector remained uncut. Possibly there was not enough SfiI to digest all-the vector present in the sample. However, it is more favourable to have 'too-little' enzyme rather than 'too-much', especially in the case of SfiI. In reactions, with the enzyme in excess of DNA sites, each site becomes loaded with SfiI tetramer, and thus preventing the protein from binding the two sites necessary for full activity. Hence, it is easier to re-digest a sample to obtain complete digestion, rather than add too much initially, and not achieve any or sufficient digestion.

Once both the vector and the SOE product were digested, they were ligated and transformed into supercompetent *E. coli* XL-1 blue cells. An antibody phage display library consisting of 5×10^3 clones was produced. This library would be considered as very small in size, as it is possible to generate libraries consisting of 10^7 or 10^8 clones. However, given the fact that the library was produced from RNA that originated from a pre-immunized mouse, it was decided to attempt panning from the library, rather-than repeating the transformation procedure to produce a larger library.

In order to select for small haptens from a phage display library it is optimal to use more than one hapten–protein conjugate. Therefore, a second AFB_1 conjugate needed to be synthesized. Amino-dextran was chosen as the carrier molecule, as previously, it has been shown to yield low background values in ELISA (Xiao *et al.*, 1995). This feature of amino-dextran is optimal for use in the panning procedure, as it is necessary to keep background signals to a minimum to ensure a non-biased selection for positive clones recognising free hapten.

Three rounds of panning were carried out on the library using two different hapten-carrier conjugates. Round 1 of panning was carried out using an AFB_1-BSA conjugate coated onto an immunotube at 50 $\mu g\,ml^{-1}$, which yielded a phage titre of 3.0×10^5 cfu ml^{-1} of phage. For round two of panning, a different hapten-carrier conjugate was used, as it was desirable to switch the carrier molecule for the second round. This ensured that non-specific phage recognising the protein (from the round 1 conjugate) were not re-selected and amplified. Instead, only phage generally recognising the hapten were selected for, thus increasing the chances of selecting and amplifying a specific phage-antibody to the hapten. As a result of this, AFB_1-dextran was used for round 2 of panning. A high concentration of 200 $\mu g\,ml^{-1}$ was used to coat the immunotube, due to the low substitution ratio on the conjugate. This yielded a phage titre of 2.8×10^7 cfu ml^{-1}, which was higher than the titre obtained from round 1. 96 random clones were picked from both rounds one and two of panning and phage was produced from them. These phage clones were then analysed for recognition to AFB_1-BSA conjugate, and subsequently, if they were positive were analysed for recognition to free AFB_1. However, results from both rounds of panning yielded no clones that recognized free AFB_1. Therefore, a third round of panning was carried out.

AFB_1-BSA conjugate was used for round 3, as the previous round ensured that non-specific 'BSA-binding' phage antibodies were removed from the selection process. As the number of rounds of panning increase, the selection for a specific phage-antibody becomes more stringent, as each round (providing different conjugates are used) ensures a reduction in the amount of non-specific phage, and greater amplification of specific phage. Round 3 gave a phage titre of 2.4×10^7 cfu ml^{-1}, which was slightly less than that obtained from round 2. 96 random clones were picked and assayed for selection to AFB_1-BSA conjugate. Seven clones showed good recognition for the conjugate, with significantly lower background levels compared to the round 2 ELISA (data not shown). The seven clones were then assayed for recognition to free AFB_1, in which six showed recognition over the range of 1–50 $\mu g\,ml^{-1}$. The panning process was highly successful, as selection of phage antibodies to small haptens is generally quite difficult.

It has been mentioned elsewhere (Moghaddam *et al.*, 2001) that selection of a large number of antibodies to low molecular weight haptens conjugated to BSA or biotinylated can often be unsuccessful for the detection of antibodies that bind the non-derivatised form of the hapten. Moghaddam *et al.* (2001) screened for phage display scFv antibodies to AFB_1 from

non-immunized human phage libraries, as well as semi-synthetic phage libraries. It was found that a large percentage of the antibodies isolated from the non-immunized (human) library specifically bound AFB_1-BSA conjugate but did not bind free AFB_1, and if so, had at least 100-times less affinity than for the BSA conjugated form. However, they did find that a percentage of the scFv antibodies that bound to both free toxin and conjugate dramatically increased, when free AFB_1 was used for elution of phage from AFB_1-BSA during the elution step of the panning. With respect to the semi-synthetic library, after three rounds of panning using the same conjugate, antibodies with high affinities to free AFB_1 were detected. This is a similar finding to what is presented in this paper, except that two different conjugates were used. However, they used AFB_1 for elution of the phage from the immunotube, which may have increased the stringency of the procedure.

Three of the clones (B3, C4, and F4) that recognized free AFB_1 were then selected for expression as soluble scFv antibodies. Restriction enzyme digestion of the pAK 100 plasmid containing the 'positive' scFv fragment was successfully carried out, as was digestion of the high expression vector pAK 400, which contains the enhanced Shine-Dalgarno sequence. Ligation of the scFv fragments into the plasmid was carried out and these were then transformed into the non-suppressor *E. coli* strain JM 83, which enhances soluble scFv expression. Analysis of the periplasmic lysates and the culture supernatants was carried out by ELISA. It was decided to concentrate on working with clone C4, as it demonstrated a good level of expression into culture supernatant and, therefore, a quicker route to obtaining and purifying the scFvs.

After scFv antibodies were produced in culture supernatant, they were concentrated five-fold and then purified using ProBond(resin, which is an IMAC-based column (Janknecht *et al.*, 1989). Immunoblot analysis was also carried out to identify the scFv antibodies. Figure 5 shows the immunoblot, where a single band can be observed for purified sample indicating the presence of scFv antibodies. No significant band was observed in the culture supernatant. This is probably because the sample was not sufficiently concentrated for the anti-FLAG antibody to detect it in the blot.

It was important to initially optimize the concentration of AFB_1-conjugate and scFv antibody for use in a competitive ELISA. A checkerboard ELISA was therefore carried out for the scFv antibody. The antibody dilution used was 1/4 with a conjugate loading density of 12.5 $\mu g\,ml^{-1}$ for the assay. A competition ELISA was optimized for the scFv antibody using these parameters. The assay was relatively sensitive, with a limit of detection of 98 $ng\,ml^{-1}$. It was also reproducible over three different days with inter-day CVs below 11.9%. Yuan *et al.* (1997) produced scFv antibodies to the mycotoxin zearalenone, and found that the soluble antibodies could detect as little as 14 $ng\,ml^{-1}$ for 50% inhibition of binding. The scFv antibodies produced by Moghaddam *et al.* (2001) could detect as little as 14 μM-AFB_1 in solution. It is clear that these antibodies are more sensitive than the scFv produced here. However, it is thought that the assay could be further optimized if a more sensitive anti-FLAG antibody could be obtained. Enzyme-labelling the anti-FLAG antibody could also improve the sensitivity of the assay, as it would reduce the number of incubation steps, as well as reduce the probability of the enzyme-labelled anti-species antibody reducing the sensitivity. Cross-reactivity studies were also carried out on the scFv antibody. Table 2 shows the results of the studies, where the scFv antibody showed high degrees of cross-reactivity, especially to $AFG_1 > AFG_2 > AFB_2 > M_1$ (in decreasing order). Moghaddam *et al.* (2001) analysed their scFv antibodies for cross reactivity using BIAcore. They found that the antibody was able to bind as effectively to AFG_1 as AFB_1. This is a similar finding to what was observed with the scFv antibody reported here. However, their scFv did not show any recognition for AFM_1 or AFM_2. It is favourable to produce specific antibodies for a particular hapten, but it could be advantageous from a regulatory viewpoint to apply this scFv antibody for the detection of total aflatoxin concentration in a sample.

When designing SPR-based immunoassays, an important factor in their design is the regenerability of the sensor-chip surface in order to carry out multiple analyses. Ideally it

should be possible to carry out 50 or more regeneration cycles on a given immobilized surface, and ligand-binding capacity should be maintained within 20% of positive control values (Wong *et al.*, 1997). Regeneration studies were carried out on the scFv anti-AFB_1 antibody for binding to an immobilized AFB_1-BSA conjugate surface. They showed that it was easily regenerated from the AFB_1-BSA immobilized surface using a 1 min pulse of 10 mM-NaOH. The binding responses were highly consistent throughout the regeneration study with a variation of binding of approximately 8%. There was no decrease in measured binding response throughout the study (i.e. cycle 1 = 244.7, cycle 49 = 244.3). The was probably due to pre-treatment of the conjugate surface with 20 1 min pulses of 10 mM-NaOH, which would have removed any loosely bound conjugate from the immobilized surface. As a result, when scFv antibody was injected over the conjugate surface, no leeching of scFv antibody-conjugate complex from the surface was observed. Therefore, a decrease in binding signal was not observed throughout the regeneration study, and the binding-regeneration pulses were true to the signal observed. The scFv antibody also displayed no non-specific binding to BSA or the CM-dextran gel surface. This is probably due to the fact that it is monoclonal in nature, and therefore recognizes only one epitope, which is present of the AFB_1 molecule.

After optimising the binding capacity and the degree of non-specific binding of the scFv antibody, the working range of detection of the BIAcore-based assay was then evaluated. The limit of quantification for the assay was 3 ng ml^{-1}, with a range of detection between 3 and 195 ng ml^{-1} (Figure 7). The intra-day CVs were between 0.16 and 1.03% (data not shown), while the inter-day CVs were between 3.35 and 19.95% (Table 3).

It can be seen that the scFv showed a high level of sensitivity for the detection of AFB_1 with a limit of detection of 3 ng ml^{-1}. The assay was also reproducible with good levels of precision, and reproducibility. However, some of the CVs were above 10%. The scFv showed very high intra-day degrees of precision with only the highest CV at 1.03%. This was probably a result of stringent pre-treatment of the immobilized surface prior to assay analysis, as well as that it was a very mild regeneration solution. This in turn gave rise to accurate binding responses during a given assay. The CVs for the scFv inter-day assay were slightly higher at certain points, indicating only a moderate degree of reproducibility. Findlay *et al.* (2000) say that since the basis of immunoassays is the antibody–antigen interaction, they may be less precise than chromatographic techniques, and as a result, existing methods with respect to degrees of assay precision are too stringent. They recommend minimal acceptance limits of 20% (25% at the limits of quantification) for precision profiles. Wong *et al.* (1997) describe the validation of an assay using BIAcore 2000 for the detection and quantification of humanized MAb in mouse serum. They examined and number of variables within the assay including degrees of precision, accuracy, specificity and sensitivity. They state that their acceptance criteria for validation is applicable to all biosensor-based immunoassays used to determine the concentration of an analyte, and indicate that results should be quantitated within 20% CV as a measure of precision. When considering these requirements, the assay presented is within the required acceptance limits and can be deemed reproducible.

The scFv antibody was also used for the detection of AFB_1 in spiked grain samples, using BIAcore. It was more sensitive for the detection of AFB_1 in grain samples compared to the model PBS assay, with a detection range between 0.75 and 48 ng ml^{-1}. The assay also showed good levels of precision (intra-day CVs: 0.17–16.53%) and reproducibility (inter-day CVs: 3.75–11.83%). Linskens *et al.* (1992) state that low concentrations of methanol extraction solvent can enhance the performance of some assays, and hence this may give a possible reason for the scFv antibody being more sensitive for the detection of AFB_1 in grain samples. Van der Gaag *et al.* (1999) developed a BIAcore-based inhibition assay for the detection of AFB_1 in spiked grain samples. They used a commercially available MAb, and directly immobilized AFB_1 onto the sensor-chip surface. The assay could detect levels as low as 0.2 ppb (ng ml^{-1}). The assay developed here is capable of detecting as low as the EU maximum residue level set for AFB_1 (3 ppb [ng g^{-1}]) in grain samples.

CONCLUSIONS

The production of an antigen-specific antibody phage display library from the RNA obtained from a mouse pre-immunized with AFB_1-BSA conjugate was successful. The stringency of the panning procedures using two toxin-carrier conjugates sequentially reduced the number of non-specific phage after each round of panning. It resulted in the highly successful isolation of seven phage display clones, six of them recognising free AFB_1 in solution. Soluble production of one of the scFv antibody clones was induced using the high expression vector pAK 400. A competition ELISA was optimized using this scFv antibody. An SPR-based inhibition immunoassay was also developed. The assay was then applied for the detection of AFB_1 in spiked grain samples. It showed a high level of sensitivity, being capable of detecting AFB_1 below the maximum residue levels allowed by the EU. The application of these antibodies for use in a 'on-line' antibody-based biosensor could have a significant impact on routine surveillance and analysis of agri-food materials.

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