Environ. Sci. Technol. **2005**, *39,* 4234−4241

Analyte Peptidomimetics Selected from Phage Display Peptide Libraries: A Systematic Strategy for the Development of Environmental Immunoassays

SOLEDAD CARDOZO,[†] ANDRÉS GONZÁLEZ-TECHERA,[†] JEROLD A. LAST,[‡] BRUCE D.HAMMOCK,[§] KARL KRAMER,[∥] AND GUALBERTO G. GONZÁLEZ-SAPIENZA*,[†]

Cátedra de Inmunología, Facultad de Química, Instituto de Higiene, UDELAR, Av. A. Navarro 3051, piso 2, Montevideo 11600, Uruguay, Pulmonary/Critical Care Medicine, School of Medicine, and Departments of Entomology and Environmental Toxicology, University of California, Davis, California 95616, and Chair of Cell Biology, Center of Life Sciences, Technical University of Munich, DE 85350 Munich, Germany

Due to their simplicity, speed, low cost, and specificity, immunoassays have become a useful tool for the analysis of environmental pollutants. Once the anti-hapten antibodies are produced, the same hapten or a related molecule is conjugated to a tracer enzyme or coating protein to set up the assay. Here we report the use of peptides that mimic the analyte as advantageous substitutes of competing haptens. These peptides, which open opportunities for innovation in the development of tracer reagents, can be selected from phage display libraries in a straightforward systematic manner. The concept was proven using assays for the herbicides molinate and atrazine as model systems. Several characteristics of the selection process that may affect the final assay were analyzed, such as the phage coat proteins fused to the peptide, the use of linear or constrained peptide libraries, the effect of the concentration of analyte used during the selection process, and the use of monoclonal or polyclonal antibodies as selector molecules. In all cases we found that the selected peptides performed with improved sensitivity as compared with the chemical hapten conventional assays, showing an analogous cross-reactivity pattern. Interestingly, the phage particles perform as robust and highly standardized assay reagents, and due to their filamentous repetitive structure, they function as sensitive multienzymatic reporters.

Introduction

The widely known advantages of simplicity, specificity, and sensitivity of immunoassays have made this technique a powerful tool in environmental analytical monitoring (*1*). The growing concern for the need for thorough control of residual levels of contaminants in diverse samples is fueling the requirement of rapid and selective quantitative methods (*2*). In view of the significant investment of time and resources involved in the development of new immunoassays, especially in the reagent preparation phase (haptens and antibodies), great efforts are directed toward the optimization of their production (*3*).

The success of immunoassay development largely depends on a critical step, the synthesis of haptens. These are analyte-mimicking substances that are conjugated to carrier proteins and used to immunize the selected animals for the generation of antibodies, the so-called immunizing haptens. Haptens are also used as competitive antigens (CpHs) in the final assay, and as such they are conjugated to coating proteins or tracer enzymes (*4*). The performance of the assay is greatly influenced by several factors related to the preparation of these conjugates, the final hapten/tracer ratio, the effect of the conjugation chemistry on the tracer enzyme activity, and the need for careful purification of the conjugate from nonconjugated reactants. In addition, some authors prefer to work with heterologous systems of haptens (i.e., the immunizing hapten and the CpH are different molecules) (*5*−*7*). When polyclonal antibodies are utilized, the use of different CpHs permits the selection of different antibody subpopulations in the serum, allowing the setup of assays with different performance characteristics with the same serum. Obtaining such compounds is usually a trial-and-error process that comprises a great deal of chemical synthesis with variable results. One approach that could be more systematic and would help to diminish the extent of chemical synthesis in the generation of haptens and facilitate the preparation of conjugated reagents for the final assay is the use of analyte peptidomimetics. Peptides can be prepared by chemical synthesis, but they can also be produced as recombinant products, which open numerous opportunities for innovative applications.

A vast repertory of candidate peptides can be expressed in phage-displayed peptide libraries, where randomly generated amino acid sequences are fused to coat proteins of the filamentous phage of the fd family (*8*, *9*). Phage libraries are enriched for specific clones by repetitive rounds of affinity selection (biopanning), which includes binding to the desired selector molecule, washing and elution, reinfection of bacteria, and growth to amplify the selected phages. Phage display peptide libraries have been extensively used primarily in the study of protein−protein interactions, epitope mapping of antibodies, selection of inhibitors or substrates for enzymes, and identification of receptor ligands (*10*). The technique has also been used to select peptide mimics of nonproteinaceous compounds, among them biotin (*11*) and carbohydrates (*12*). Recently, this approach was used to find peptides that can mimic the mycotoxin deoxyvalenol (DON) and can be used in immunoassays (*13*).

In this work we investigate the use of phage-borne peptides as substitutes of CpH in enzyme-linked immunosorbent assays (ELISAs). The concept was explored utilizing two assays for the herbicides molinate and atrazine as model systems. The versatility of this approach was demonstrated using libraries that express linear or cyclic peptides fused to different phage coat proteins, and using either monoclonal or polyclonal antibodies as selector molecules. Interestingly, the phage particle itself can be conveniently used as a stable and robust assay reagent, which may be easily standardized because phage can be cloned and propagated indefinitely (*14*).

* Corresponding author phone: (5982) 4874334; fax: (5982) 4874320; e-mail: ggonzal@fq.edu.uy.

[†] UDELAR.

[‡] Pulmonary/Critical Care Medicine, School of Medicine, University of California.

[§] Departments of Entomology and Environmental Toxicology, University of California.

[∥] Technical University of Muenchen.

10.1021/es047931l CCC: $30.25
Published on Web 05/04/2005

Materials and Methods

Antibodies and Herbicide-Related Compounds. Molinate and the thiocarbamate compounds used in the inhibition assays were gifts from Stauffer Chemical Co. Thiobencarb was a gift from Chevron Chemical Co. The molinate derivatives *S*-2-carboxyethyl hexahydrozepine-1-carbothioate (**3a**) and *S*-2-(*p*-aminophenyl)ethyl hexahydroazepine-1-carbothioate (**7b**) and the rabbit polyclonal serum anti-molinate (pAb 245) were obtained previously in Dr. Hammock's laboratory (*15*). The hapten **3a** conjugated to ovalbumin (**3a**−OVA) was coupled to cyanogen bromide (CNBr)-activated Sepharose 4B (Sigma) and used to purify anti-molinate-specific γ globulins from pAb 245. Development of the monoclonal anti-molinate antibody (mAb 14D7) has been described in detail previously (*16*). Atrazine and the related triazine compounds were donated by Ciba-Geigy and Du Pont. The triazine derivative for tracer synthesis, 4-chloro-6-(isopropylamino)-1,3,5-triazine-2-(6-aminohexanecarboxylic acid) was kindly provided by the group of Rolf Schmid, Institute of Technical Biochemistry, University of Stuttgart, Germany. The production of the monoclonal antibody raised against atrazine (mAb K4E7) was described previously (*17*).

Phage Display Peptide Libraries and Biopanning. The phage display peptide libraries used in the selection procedure were a kind gift from Dr. Peter Schatz (Affymax Research Institute, Palo Alto, CA) and consisted of (i) a linear library, 141, expressing an 8-mer random linear peptide (X8-ASGSA-pIII, size 6×10^8 equiv) (pIII = coat protein encoded by gene III on a filamentous phage) and (ii) a constrained library, 549−553, expressing 7−10- or 12-mer random residues flanked by two cysteine residues (ASGSA-C(X)7−10,12CG(P)6G-pIII, size 1.0×10^9 equiv), both constructed in the phagemid vector pAFF2 (*18*), expressing the random peptides fused to the N-terminal end of the coat protein pIII, and (iii) a linear library, 2158 (X11-((G)4S)3)-pVIII, size 1.05×10^{11} equiv) (pVIII = coat protein encoded by gene VIII on a filamentous phage), constructed in the phagemid vector p8V2 (*19*), expressing an 11-residue random linear peptide fused to the N-terminal end of the coat protein pVIII. For the selection procedure, Nunc-Immuno modules were coated with the mAbs or the affinity-purified γ globulins from pAb 245, at 1 μg/well, 10 μg/mL in phosphate-buffered saline (PBS) overnight at 4 °C. After a blocking step of 1 h at 37 °C with 1% bovine serum albumin (BSA) in PBS, phage particles in a number equal to 100−1000 library equivalents ($\cong 10^{10}$ transducing units) diluted in 600 μL of PBS−1% BSA were added to six-well plates and incubated for 2 h at 4 °C. Unbound phages were removed by 10 washes with cold PBS containing 0.05% Tween 20 (PBST). Bound phages were eluted by incubation with PBS containing 1 or 0.1 ng/mL molinate or atrazine, respectively, for 30 min at room temperature. After the phages were washed with a 10 ng/mL concentration of the appropriate pesticides, a second step of elution was performed with 100 ng/mL for both pesticides. The eluted phages (600 μL) were added to 10 mL of log-phase *Escherichia coli* ARI 292 (Affymax Research Institute, Palo Alto, CA) cells and amplified in Luria−Bertani (LB) medium containing 0.25% K_2HPO_4, 0.1% $MgSO_4$, 0.1% glucose, and 100 μg/mL ampicillin to $OD_{600} = 0.4$. Helper phage M13KO7 (Pharmacia Biotech) at a multiplicity of infection of 10:1 was added. After a period of 30 min at 37 °C without shaking, arabinose and kanamycin were added to a final concentration of 0.02% and 40 μg/mL, respectively, and the cultures incubated overnight at 37 °C with vigorous shaking. Phages from liquid cultures were obtained by clearing the supernatants by centrifugation at 12000*g* for 15 min, precipitated with 0.2 volume of 20% poly(ethylene glycol) 8000−2.5 M NaCl (PEG−NaCl) on ice for 1 h, and centrifuged as above. Phage pellets were resuspended in 600 μL of sterile PBS and titrated in ARI 292. A number of 10^{10} transducing units were used for the next round of selection. This panning protocol was then repeated twice. After three rounds of panning, individual amplified phage clones were tested for their ability to bind to the antibodies by a direct ELISA (see below). Positive clones were further selected on the basis of their performance in the competitive assay described below, and were submitted for DNA sequencing (Division of Biological Sciences (DBS) Automated DNA Sequencing Facility, University of California, Davis). Primers used were ON3 (cgatctaaagttttgtcgtct) or ON891 (tgaggct-tgcaggggtc) for peptides expressed as fusions with pIII or pVIII, respectively.

Phage Direct ELISA. A 100 μL sample of the corresponding antibody diluted to a concentration of 5 μg/mL in coating buffer (100 mM $NaHCO_3$, pH 9.6) was dispensed into the wells of 96-well microtiter plates (Nunc-Immuno Plate MaxiSorp). After overnight incubation at 4 °C, the plates were blocked with 1% BSA in PBS and washed five times with PBST, and then 100 μL/well of an overnight culture of individual amplified phage clones was dispensed. Control wells coated with an unrelated antibody or with 1% BSA were always included to rule out nonspecific binding. The microtiter plates were incubated for 1 h at room temperature with gentle rocking. After a washing step with PBST, 100 μL of a 1:5000 dilution of horseradish peroxidase (HRP)-labeled anti-M13 monoclonal antibody (Pharmacia) in PBST was added to each well. After 1 h, the plates were washed, and 100 μL of the peroxidase substrate (0.4 mL of a 6 mg/mL DMSO solution of 3,3′,5,5′-tetramethylbenzidine (TMB) and 0.1 mL of 1% H_2O_2 in water in a total of 25 mL of 0.1 M citrate acetate buffer, pH 5.5) was dispensed into each well. The enzymatic reaction was stopped after 15−20 min by the addition of 50 μL of 2 M H_2SO_4, and the absorbance at 450−600 nm was read in a microtiter plate reader (Multiskan MS, Labsystems).

Phage Suspensions for Competitive ELISA. Individual amplified phage clones were obtained as described above. After two steps of precipitation with PEG−NaCl, the phage particles were suspended in 1/50 volume of the original culture volume in PBS, which was supplemented with the complete protease inhibitor cocktail of Roche Diagnostics and sodium azide, 0.05%. The preparations were filtered through a 0.22 μm filter and stored in aliquots at 4 °C and −20 °C.

Competitive ELISA. Serial dilutions (50 μL/well) of the pesticide standards in PBST were dispensed into microtiter plates coated and washed as above, followed by addition of 50 μL/well of the adequate dilution of the phage suspension (previously optimized by checkerboard titration). After a 1 h incubation period at room temperature, the plates were washed and developed as previously described. Standard curves were normalized by expressing experimental absorbance values (B) as $(B/B_0) \times 100$, where B_0 is the absorbance value at zero pesticide concentration. Normalized values were fitted to a four-parameter logistic equation using Genesis Lite 3.03 (Life Sciences (UK) Ltd.) package software.

Microtiter Plate ELISA for Atrazine Using the Chemical Hapten. The triazine derivative 4-chloro-6-(isopropylamino)-1,3,5-triazine-2-(6-aminohexanecarboxylic acid) was coupled to HRP as described previously (*17*). For the competitive assay with atrazine, microtiter plates were precoated overnight with 100 μL/well of goat anti-mouse IgG (2.5 μg/mL in coating buffer) at 4 °C. After a washing step with PBST, 100 μL of 300 ng/mL mAbK4E7 was added to each well, and the plates were incubated for 1 h at 37 °C. The plates were washed five times, and then 50 μL of a 1:20000 dilution of the HRP tracer and 50 μL of different atrazine dilutions in PBST were added to each well (working dilutions were previously optimized by checkerboard titration). After a 1 h incubation period at room temperature, the plates were washed and developed as previously described.

Assay Cross-Reactivity. The selectivity of the union between the phage-borne peptide and the mAbs was characterized by determining the cross-reactivity with some commercial pesticides and other related compounds. Inhibition curves in the 0−100000 ng/mL range were used in the competitive ELISA. After the data were normalized, the molar compound concentrations that caused 50% inhibition (IC_{50}) were used to express the cross-reactivity according to the equation

$$\% \text{ cross-reactivity} = 100[IC_{50}(\text{analyte})/IC_{50}(\text{cross-reacting compound})]$$

Transfer of Peptides between Phage Display Systems. Peptide HPWEDRQSAFL, originally selected from library 2158, as a fusion with pVIII, was transferred to the phagemid vector pAFF2, a pIII system expression. A DNA oligonucleotide coding for this peptide was synthesized with the sequence 5′-Phos/CTC TCA CTC CCA TCC GTG GGA GGA CCG TCA GAG TGC TTT TTT GGG CGG TAC CGC TTC TGG ATC TGC TAC TAG TAC TGT TGA AAG TTG T-3′. Two "half-side" oligonucleotides complementary to its 5′ and 3′ ends (5′-GGA GTG AGA GTA GA-3′ and 5′-CTT TCA ACA GT-3′) were synthesized to facilitate annealing to the pAFF2 vector. In a similar way, peptide CSSMGQWAKC, originally selected from library 549−553, as a fusion with pIII, was transferred to the phagemid vector p8V5, a pVIII system expression. A DNA oligonucleotide coding for this peptide was synthesized with the sequence 5′-Phos/CAC GCA GCG TCT GGG TCC GCG TGT AGT AGT ATG GGT CAG TGG GCT AAG TGT GGC CCT CCT CCT CCT CCT CCT GGC GAC GAT CCC AGC AAG. Two half-side oligonucleotides complementary to its 5′ and 3′ ends (5′-GACGCTGCGTGAGCA-3′ and 5′-CTGGGAT-CGTCGC-3′) were synthesized to facilitate annealing to the p8V5 vector. The sets of oligonucleotides were annealed as previously described (*8*). The fragments were ligated, transformed in electrocompetent ARI 292, and plated in agar plates with ampicillin. Isolated clones were grown and infected with helper phage M13KO7 to produce phage particles and used as described before.

Results

Pesticide-Mimicking Peptides Can Be Selected from Different Phage Display Library Formats. To explore the feasibility of finding suitable peptides that can be used as competitive haptens in immunoassays, we used two model systems, namely, the assay for molinate based on the use of mAb 14D7 (*16*) and an atrazine assay that utilizes mAb K4E7 (*17*). Both mAbs were immobilized in polystyrene ELISA plate wells, and used for library selection. After three rounds of panning, the reactivity of each of the individual phage supernatants was tested by ELISA. Positive supernatants showing inhibition by the pesticides in a competitive ELISA were submitted for sequencing. In general, it was possible to obtain a large percentage of phage-borne peptides that bind specifically to the mAbs from the different libraries, including phage expression in pIII and pVIII and linear or constrained peptides (Tables 1 and 2). While the linear libraries yielded a limited number of sequences, probably due to overpanning, the disulfide-constrained library produced a large panel of related sequences with both mAbs. In general, there was a correlation between the concentration of pesticide used for elution during the panning experiments and the peptides selected, suggesting that peptides with different affinities were selected. Indeed, for both mAbs, it was possible to group the panel of sequences selected from the constrained libraries into two families of related peptides, according to the presence of the consensus residues shown in bold in the tables. That is the case of the motif GxWxK that was predominantly selected by elution with 100 ng/mL molinate (Table 1). Similarly, in the selection performed with mAb K4E7, four out of six clones selected with 0.1 ng/mL atrazine contained the LR motif, while six out of eight clones eluted with 100 ng/mL atrazine exhibited the RL motif. This was even clearer in the experiment performed with the linear library 2158 using mAb 14D7, where all 10 clones analyzed from the pool selected with 1 ng/mL molinate had the HPWEDRQSAFL sequence, while all 10 clones selected with 100 ng/mL contained the DRYLELQDLYF peptide.

TABLE 1. Amino Acid Sequences of Selected Clones Isolated with mAb 14D7 Using Different Concentrations of Molinate[a]

Library	Eluted with 1 ng/ml	Eluted with 100 ng/ml	Family
549-553 (pIII)	CTDR**GGWTK**C CNLMG**SWAK**C	CAHM**GGWSK**C CSSMG**QWAK**C CMLF**GGWSK**C (2) CNLMG**SWAK**C CLGFEY**GGWSK**HDC	**GxWxK**
	C**WE**GDMWLNVHC CET**WLTWE**LC (3) CSV**WRTWE**LC	CDSSL**WFTWE**C CSV**WRTWE**LC	**WxTWE**
2158 (pVIII)	HPWEDRQSAFL (10)	DRYLELQDLYF (10)	-

[a] The number of isolates bearing the same sequence is indicated in parentheses. The consensus residues are shown in bold.

TABLE 2. Amino Acid Sequences of Selected Clones Isolated with mAb K4E7 Using Different Concentrations of Atrazine[a]

Library	Eluted with 0.1 ng/ml	Eluted with 100 ng/ml	Family
549-553 (pIII)	CERDLFG**LR**C CMGA**LRW**LPNC CRVDH**LIW**DPLGRC CNLP**LR**VNDC	CGSSP**LRW**HPLYDC (2)	**LRW**
	CNHTEIT**RL**SYC CVGPLGGQW**ND**RLC	CLDT**P**H**RL**QYC CKDFVRVH**P**S**R**SVC (2) CST**HP**S**RL**EYC CGA**PERL**SYC CWHVGFW**NERL**C	**P/NxRL**
2158 (pVIII)	RVPRECMSILI (3) RLDLLLLETCL (2)	Not performed	-
141 (pIII)	**LSWDWLGR** (4) **LWRDWLGR**	**LSWDWLGR** (7)	**LxxDWLGR**

[a] The number of isolates bearing the same sequence is indicated in parentheses. The consensus residues are shown in bold.

The overall analysis of the consensus sequences of the peptide families displayed in Tables 1 and 2 shows that only a limited set of amino acids participate in analyte mimicking, with a dominant presence of tryptophan. In most of the cases, this bulky aromatic residue is probably needed to occupy the hydrophobic pocket that is normally found in the antigen-binding site of anti-hapten immunoglobulins. However, the other residues of the consensus and the proper spacing among them also appear to be critical for binding, because despite some similarities among the consensus sequences isolated with the two antibodies, we did not observed cross-reactivity between them.

Phage-Borne Peptides Can Be Conveniently Used in the Setup of a Competitive Immunoassay for Pesticides. Phage particles purified and stabilized as described in the Materials and Methods were titrated and used as a reporter system for immunoassay setup. The phage suspensions prepared in this way remained stable at 4 °C, and once titrated could be stored for months without appreciable loss of titer. The analysis of several clones revealed minor differences in the final sensitivity of the assay, regardless of the library and conditions used to select the clone. This is exemplified in Figure 1, which shows the competition curves obtained with four representative phage clones of the peptide families selected with mAb K4E7 using low and high concentrations of atrazine from disulfide-constrained and linear libraries. The sensitivity of the assay is therefore set by the antibody/analyte system, and did not change with the expected differences in the

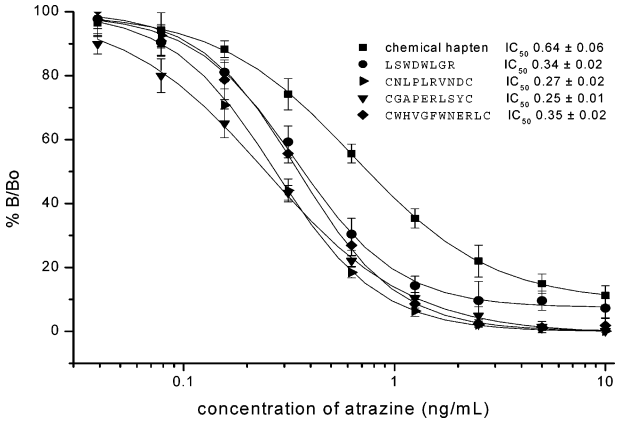


FIGURE 1. Atrazine competitive assay using phage-borne peptides and the conventional chemical reporter. Phage-borne peptides and the HRP-conjugated atrazine chemical hapten (squares) were incubated in plates coated with mAb K4E7, with different concentrations of atrazine. Peptide LSWDWLGR (circles) derives from library 141, while peptides CNLPLRVNDC (right triangles), CGAPERLSYC (inverted triangles), and CWHVGFWNERLC (tilted squares) derive from cyclic library 549−553. Bound phage peptides were detected using HRP-conjugated anti-M13 mAb, and the absorbance was read at 450/600 nm. Values are the mean of two independent experiments with three replicates each. Maximum absorbance readings were 1.1 for the chemical hapten and 1.5, 1.5, 1.0, and 1.2 for the phage-borne peptides in the order cited.

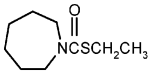
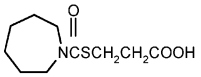
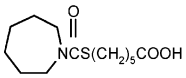
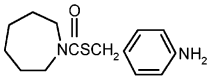
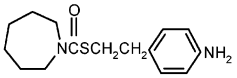
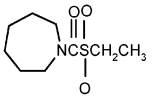
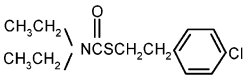
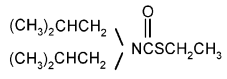
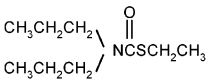
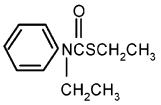
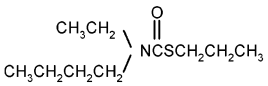
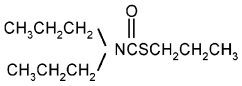
affinity of the peptides for the antibody, as could be anticipated according to the mass action law when monoclonal antibodies are used. However, the sensitivity of the phage assay was to some extent improved as compared to that attained using the chemical hapten conjugated to the reporter enzyme, and the possible reason for that is analyzed later. Analogous overlapping curves were obtained with various peptidomimetics selected on mAb 14D7 by elution with molinate (not shown). The IC_{50} values attained with these peptides were in the 40−50 ng/mL range, similar to the value reported previously (*16*) using a chemically synthesized hapten (69.2 ± 1.4 ng/mL molinate). Thus, in the two models explored, phage-borne peptides were found that could be successfully used as competitive haptens, producing assays with IC_{50} values similar to or improved over those obtained with the conventional heterologous chemical haptens.

The Cross-Reactivity Pattern of the Selected Peptides Is Similar to That Obtained with the Conventional Assay. The specificity attained with the peptides was assessed using a panel of closely related carbamate pesticides and molinate haptens **3a**, **3b**, **7a**, and **7b**. Table 3 shows the IC_{50} values obtained with two molinate-mimicking peptides, CETWLTWELC and CSSMGQWAKC. Both phage-borne peptides performed in a similar manner. Significant cross-reactivity was only observed with the closely related haptens **3a**, **3b**, **7a**, and **7b**, but not with other carbamate pesticides. Overall, the specificity obtained with the peptides was very similar to the cross-reactivity reported for the conventional assay set up with the use of compound **7b** as the CpH conjugated to conalbumin (*16*). Similarly, Table 4 exhibits the percentage of cross-reactivity obtained with different triazines when the peptide LWRDWLGR, or the conventional hapten tracer, was used to set up the assay. Once more, the specificity attained was similar in both formats, reinforcing the viability of using the phage-borne peptide as a surrogate hapten.

The Nature of the Filamentous Phage Particle Represents an Advantageous Feature for Its Use as a Reporter System in Competitive Immunoassays. There are two common ways of displaying fusion peptides on a phage; the most used vectors utilize the minor coat protein, pIII, or the major coat protein, pVIII, for such fusions. When the recombinant fusion is encoded in a phagemid system, which requires the use of a wild-type helper phage to produce hybrid phage particles, 0−5 copies of the peptide can be displayed in pIII and up to 2400 copies in pVIII. The average valency on the phage particle can be modulated by controlling the expression of the phagemid cassette. The conditions used in this study promote, mostly, monovalent expression in pIII libraries, and the display of a few hundred copies in pVIII (*20*). A schematic representation of competitive immunoassays set up with phage particles obtained from pIII and pVIII libraries is shown in Figure 2. In the conventional format, roughly one molecule of analyte displaces one molecule of reporter enzyme. However, after addition of the secondary antibody, the phage particle (7 nm wide by 900−2000 nm in length) works as a multienzymatic reporter system, and in the case of phage particles displaying peptides in pIII, the ratio of reporter enzyme to peptide is highly favorable. Therefore, each molecule of analyte produces a significant displacement of enzymatic activity, with the consequent improvement of the assay sensitivity. Conversely, expression in pVIII represents a less favorable situation, due to the fact that several peptide−antibody interactions should be competed to release the phage particle. This was observed when the assay set up for molinate using the peptides CSSMGQWAKC (from the pIII library) and HPWEDRQSAFL (from the pVIII library) were compared. As expected, the latter produced a larger IC_{50} (160 versus 56 ng/mL), Figure 3. To confirm that this difference was due to the display format and not to the peptide sequence displayed, the CSSMGQWAKC and HPWEDRQSAFL sequences were cross-cloned in pVIII and pIII, respectively, and the resulting phage particles were used in the assay. As seen in Figure 3, under these conditions, the changing of CSSMGQWAKC expression from pIII to pVIII caused a variation of IC_{50} from 56 ± 4 to 120 ± 11, while expression of HPWEDRQSAFL in pIII brought the IC_{50} from 160 ± 19 to 28 ± 4 ng/mL.

Polyclonal Antibodies Can Also Be Used as Selector Molecules To Identify Pesticide-Mimicking Peptides. Because this study is focused in the development of a rapid and systematic method to find competitive haptens for the development of immunoassays, we also explored the feasibility of using polyclonal antibodies as target molecules for the selection of suitable peptide candidates. Polyclonal antibodies are easily produced, and since hyperimmune sera normally contain high affinity antibodies, they are routinely used in immunoassay development. Anti-molinate-specific antibodies were affinity purified from a hyperimmune serum on immobilized hapten **3a**−OVA, and the purified antibodies were used to pan the 549−553 library using 10 ng/mL molinate for elution. The inset in Figure 4 shows the peptide sequences of seven selected clones. As expected, on the basis of the polyclonal nature of the selector molecules, there were no clear consensus sequences, though many sequences could be aligned according to a common **F**(x)2/3**W** motif. The high occurrence of bulky hydrophobic residues in these peptides is in agreement with the abundance of these residues in the sequences selected with mAb 14D7, suggesting that peptide mimicking of molinate requires the participation of aromatic rings. The clone DWFYEWTV, exhibiting the highest titer, was selected and used to set up the competition assay using the anti-molinate antibodies. Figure 4 shows the assay performance when the phage-borne peptide and the conventional chemical hapten were compared. Once more it was possible to isolate a peptide that allowed the setup of

TABLE 3. Cross-Reactivity of Different Thiocarbamates and Related Compounds in a Competitive ELISA Set Up with Two Phage-Borne Peptides[a]

Compound		CETWLTWELC		CSSMGQWAKC	
		IC_{50} ng/mL	% cross-reactivity	IC_{50} ng/mL	% cross-reactivity
molinate		44	100	56	100
3a		35	160	49	136
3b		14.9	444	83.5	100
7a		111	57	42.8	188
7b		87	77	154	55
molinatesulfone		0	0	0	0
thiobencarb		0	0	0	0
butylate		2066	5.8	1059	6
EPTC		1954	2.5	1838	3
cycloate		2407	2.1	2596	2.5
pebulate		2148	2.2	2067	3
vernolate		2316	2.1	1723	3.5

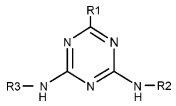
[a] Microtiter plates were coated with 100 μL of 14D7 (5 μg/mL). The appropriate dilutions of each phage-borne peptide were incubated with several concentrations of the compound listed. The highest concentration used for compounds listed between molinate and **7b** was 1000 ng/mL, and for those listed below it was 100 000 ng/mL. All data are the mean value of two independent experiments. A value of 0% cross-reactivity means that there was no inhibition at the highest concentration tested.

a sensitive assay, with an improved IC_{50} (73 ± 9 versus 199 ± 22 ng/mL).

Discussion

In this work we explored the concept that phage display libraries could be a useful source of peptidomimetics for the substitution of conventional CpHs in immunoassays for small molecules. A priori, there are several potential advantages in this approach: First, phage display peptide libraries are readily available, and this technology can be easily incorporated in most laboratories. Second, the selection of phage-borne peptides using antibodies as selector molecules is a fast and straightforward technique. Third, the phage particles could be used as final assay reagents, avoiding the conjugation step related to the use of conventional CpHs. Additionally, as discussed before, there is a potential advantage in the use of heterologous assays when polyclonal antibodies are used, and many researchers routinely prepare a CpH panel to optimize the assay sensitivity. Obtaining the right compounds is usually a trial-and-error process that requires a significant effort of chemical synthesis and the preparation of a panel of hapten−carrier conjugates to find the proper CpH antibody

TABLE 4. Cross-Reactivity of Different *s*-Triazines in a Competitive ELISA Set Up with the Phage-Borne Peptide LSWDWLGR or the Chemically Competing Hapten[a]



compd	R1	R2	R3	chemical hapten IC_{50},[b] M × 10^{-8}	% cross-reactivity	phage-borne peptide LSWDWLGR IC_{50},[c] M × 10^{-8}	% cross-reactivity
atrazine	Cl	C_2H_5	$CH(CH_3)_2$	0.29	100	0.16	100
simazine	Cl	C_2H_5	C_2H_5	0.6	48	0.2	80
propazine	Cl	$CH(CH_3)_2$	$CH(CH_3)_2$	0.25	116	0.09	177
cyanazine	Cl	C_2H_5	$C_2N(CH_3)_2$	0.32	91	0.18	88
ametryn	SCH_3	C_2H_5	$CH(CH_3)_2$	252	<0.1	128	<0.1
simetryn	SCH_3	C_2H_5	C_2H_5	257	<0.1	166	<0.1
prometryn	SCH_3	$CH(CH_3)_2$	$CH(CH_3)_2$	117	<0.2	121	<0.1
terbutryn	SCH_3	C_2H_5	$C(CH_3)_3$	181	<0.2	199	<0.1

[a] The appropriate dilutions of phage-borne peptide or HRP−atrazine tracer were incubated with serial dilutions of the compound listed. The highest concentration used for atrazine, simazine, propazine, and cyanazine was 10 ng/mL, and for the others it was 1000 ng/mL. All data are the mean value of two independent experiments. [b] Microtiter plates (previously precoated with goat anti-mouse IgG) were incuabated with 100 μL of 300 ng/mL mAb K4E7. [c] Microtiter plates (previously precoated with goat anti-mouse IgG) were incuabated with 100 μL of 5 μg/mL mAb K4E7.

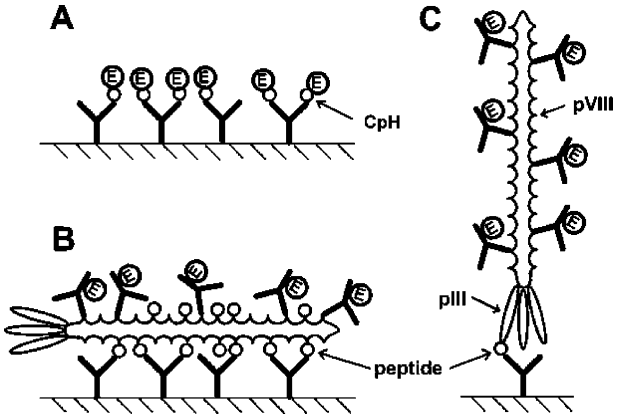


FIGURE 2. Schematic comparison of the conventional and phage competitive immunoassays. In all formats the antibody specific for the pesticide has been immobilized in the ELISA plate. Panel A depicts the conventional competitive immunoassay where the chemical hapten is directly coupled to the reporting enzyme. In panels B and C, the signal is provided by the large number of enzyme−antiphage antibodies bound to the major phage coat protein. When the pesticide-mimicking peptide is fused to pVIII (panel B), there is a multivalent interaction with the immobilized antibodies. On the contrary, expression in pIII results in a monovalent interaction of the phage particle with the antibody. In the latter case, a great deal of enzymatic activity is released by displacement of the phage particle with a single molecule of analyte.

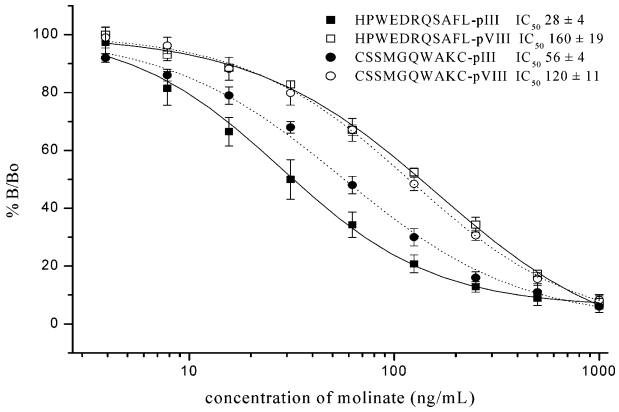


FIGURE 3. Effect of the pIII and pVIII display on assay sensitivity. A molinate competitive assay was set up as described using phage-borne peptides CSSMGQWAKC, displayed in pIII (closed circles) or in pVIII (open circles), and HPWEDRQSAFL, displayed in pIII (closed squares) or in pVIII (open squares). Values are the mean of two to three independent experiments with three replicates each. Maximum absorbance readings were 1.2, 0.8, 0.6, and 1.2 for peptides CSSMGQWAKC and HPWEDRQSAFL displayed in pIII and pVIII, respectively.

interaction. The phage selection process ends up with a panel of candidate peptides, which differ in their affinity for the antibody, and could be directly assessed for assay performance.

To prove this concept, we worked with two assays for herbicides. The assays differ in that the molinate assay is moderately sensitive, while the atrazine assay is highly sensitive. Initially, our work was focused on the use of monoclonal antibodies, because due to their single specificity, they constitute simpler probes, which simplifies the analysis of the selected sequences and the interpretation of the role of the phage expression systems. In all panning experiments it was possible to obtain phage-born peptides that bind specifically to the antibodies and allow the setting up of sensitive competitive ELISAs for quantification of atrazine and molinate. Several characteristics of the selection process that may influence the performance of the selected peptides were analyzed, including (a) the use of linear versus constrained libraries, (b) the selection under low or high concentration of analyte, (c) the display format (pIII versus pVIII), and (d) the feasibility of using mAb and polyclonal antibodies as selector molecules. In general, after three rounds of panning, the libraries containing disulfide-constrained peptides yield the most diverse panel of peptides (Tables 1 and 2), and two families with different consensus sequences were identified. The relative number of peptide sequences that belong to each of these families differed according to the concentration of analyte used for elution in the panning experiments, suggesting that clones with different affinities were selected, as has been observed in other systems (*21*).

When representative clones of each family were evaluated, no differences in the assay sensitivity were observed, and similar results were also attained when the linear peptides selected with low and high concentrations of herbicides were tested. Despite minor differences, it is clear that, after optimization, the sensitivity and degree of cross-reactivity of the assay are determined by the antibody/analyte system

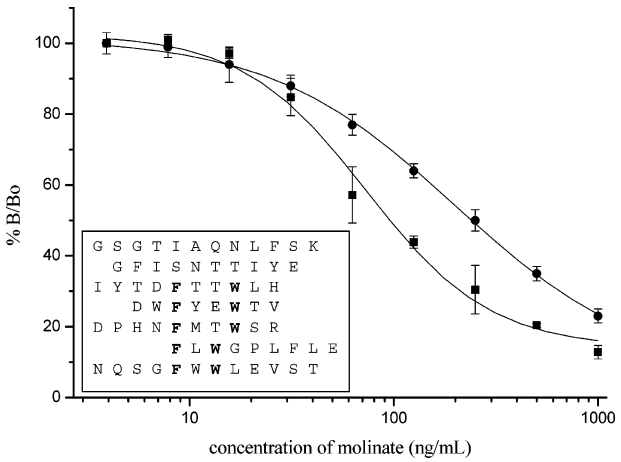


FIGURE 4. Molinate competitive assay using a phage-borne peptide selected with polyclonal antibodies and the conventional chemical hapten. The molinate competitive assay was set up as described using phage-borne peptide DWFYEWTV (squares) and was compared with the conventional assay as described previously (*15*) (circles). Values are the mean of two independent experiments with three replicates each. Maximum absorbance readings were 0.6 and 1.1, respectively. The inset shows the sequences of selected clones.

and not by the characteristics of the selected peptide. In the case of monoclonal antibodies this can be anticipated on the basis of thermodynamic considerations, and has practical considerations because once any positive clone has been selected it could be directly used for assay optimization. In the case of polyclonal antibodies, the selection of a panel of candidate peptidomimetics would offer additional advantages, because the different families of peptides, or even individual family members, are selected by different antibody subpopulation of the serum. These subpopulations are all reactive against the immunizing hapten, but react with the analyte with different affinities and possess different patterns of cross-reactivity with related compounds. The preferential use of these subpopulations by using a CpH that differs from the immunizing hapten constitutes the basis of the heterologous approach. Thus, as we demonstrated with the anti-molinate hyperimmune serum, when polyclonal antibodies are the option, they can be used in the panning experiment to obtain a panel of CpH peptidomimetics that can be directly used for assay optimization. This is a systematic strategy that avoids the laborious alternative of chemical synthesis.

While only a minor effect of the peptide sequence on assay sensitivity was observed when monoclonal antibodies were used, the display format does have a major impact. As seen in Figure 2, the phage particle behaves as a multienzymatic tracer, and the most advantageous use of this feature occurs when the analyte peptidomimetic is expressed in pIII. The filamentous nature of M13 phages offers a large surface ($(3.5-7.7) \times 10^6$ Å^2), which by reaction with the secondary antibody can be loaded with a large number of tracer enzyme molecules, and this may be the main reason for the improved sensitivity obtained with those peptides. Additional strategies that increase the enzymatic activity associated with the phage (i.e., its biotinylation) could intensify this effect. The use of phage particles as competitive tracers in ELISAs offers some additional advantages. Due to the linkage between the displayed peptide and the DNA information, once a suitable candidate is found, it can be reproduced and propagated indefinitely as a defined reagent. Recently, there have been some reports on the use of the phage particle itself as an effective tool for the detection of various biological agents (*22*). In our applications, we have found that the viral particles can be utilized directly as assay reagents and, once prepared, can be stored refrigerated for prolonged periods of time with stable titers, as has been found by other researchers (*23*). Because the phage surface exposes thousands of reactive amino groups, it can be easily conjugated with fluorescent labels (*24*) or reporter enzymes, avoiding the use of a secondary antibody and therefore shortening the assay time. If required for safety reasons, truncated forms of pIII (lacking the N1 and N2 N-terminal domains) can be engineered, which will render noninfective phage particles.

Finally, the availability of analyte peptidomimetics goes beyond the use of the phage particles as assay reagents and opens opportunities for innovative applications; for instance, using recombinant technology, the peptide sequence can be fused to the gene of a label enzyme to create highly reproducible recombinant tracers. The peptide can also be genetically engineered in the vicinity of the active site of a tracer enzyme to obtain an antibody-mediated modulation of the enzymatic activity, which can be useful for the development of homogeneous noncompetitive immunoassays (*25*). None of these developments are possible with the use of the chemical hapten.

In summary, our data demonstrate that the selection of pesticide peptidomimetics from phage display peptide libraries can be a useful alternative for immunoassay development. Though the initial synthesis of the immunizing hapten will still be required, once the monoclonal or polyclonal antibodies are produced, it offers a fast and systematic strategy for the preparation of tracer reagents for sensitive assays. Moreover, the selected phage-borne peptide constitutes a defined and reproducible reagent that can be directly used without the need for additional conjugation steps. A further development of this approach may include the use of the free peptide as a surrogate standard, which will produce a nonhazardous assay, a relevant issue in many applications related to environmental pollutants, which are frequently highly toxic.

Acknowledgments

This work was supported in part by NIH Fogarty International Center Grant TW05718, the NIEHS Superfund Basic Research program, Grant P42 ES04699, and the NIEHS Center, Grant P30 ES05707. We thank Dr. Peter Shatz, Affymax Research Institute, Palo Alto, CA, for his generous donation of the phage libraries used in this study.

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Received for review December 31, 2004. Revised manuscript received March 30, 2005. Accepted March 31, 2005.

ES047931L