

FINAL REPORT
U.S. DEPARTMENT OF ENERGY
ENGINEERED ANTIBODIES FOR MONITORING OF POLYNUCLEAR AROMATIC HYDROCARBONS

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PROJECT NUMBER: 54546

GRANT NUMBER: FG07-96ER62316

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PROJECT DURATION: Four years (15 Sept. 1996 – 14 Sept. 1999, and
no-cost extension, 15 Sept. 1999 – 14 Sept. 2000)

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EXECUTIVE SUMMARY

This project was undertaken to fill needs in DOE's human and ecosystem health effects research, site remediation, rapid emergency response, and regulatory compliance monitoring programs.

DOE has greatly stimulated development and validation of antibody-based, rapid, field-portable detection systems for small hazardous compounds. These range from simple dipsticks, microplate enzyme-linked immunosorbent assays (ELISAs), and hand-held colorimeters, to ultrasensitive microfluidic reactors, fiber-optic sensors and microarrays that can identify multiple analytes from patterns of cross-reactivity. Unfortunately, the technology to produce antibodies with the most desirable properties did not keep pace. Lack of antibodies remains a limiting factor in production and practical use of such devices. The goals of our project were to determine the chemical and structural bases for the antibody-analyte binding interactions using advanced computational chemistry, and to use this information to create useful new binding properties through *in vitro* genetic engineering and combinatorial library methods.

The target analytes were polynuclear aromatic hydrocarbons (PAHs), which pose significant hazards in numerous DOE problem areas. Reliable multi-analyte immunoanalysis of PAHs could achieve very significant savings of money and time. However, structural and chemical properties make PAHs inherently cross-reactive and difficult to distinguish with antibodies. Two separate approaches were taken: (a) existing monoclonal antibodies (MAbs) that bound certain PAHs were subjected to successive rounds of knowledge-based mutation and selection *in vitro*, and (b) attempts were made to select useful PAH-specific antibodies from two very large combinatorial phage-display libraries. A set of haptens representing eleven of the most important 2-, 3-, 4, and 5-ring PAHs was synthesized in Dr. Li's and Dr. Karu's laboratories. Dr. Karu's laboratory did the molecular cloning, selection from phage display libraries, and *in vitro* mutagenesis. Dr. Li's group extensively characterized performance of the antibodies in immunoassay and immunoaffinity applications, and developed procedures to recover PAHs and their metabolites from biological and environmental samples. Dr. Roberts's lab created highly accurate models of the antibody binding sites, applied advanced computational methods to determine the shapes and charge distributions of the analytes and haptens, and then used computational docking programs to determine the position of bound haptens and analytes. From these results, they determined which

amino acid side chains were involved in binding, and which parts of the antibody could be altered to produce new properties. Communications between our three laboratories were frequent, and proved essential throughout the project.

The major accomplishments and their significance are summarized as follows:

- a) A comprehensive set of PAH haptens with 2, 3, 4, and 5 rings was developed. To our knowledge, this was the largest set of published, openly available PAH haptens to date. All were prepared using simplified procedures that involved either a Friedel-Crafts or a Wittig reaction. These procedures can also be used to synthesize larger sets of haptens for PAHs and PAH analogs.
- b) Simple competition ELISAs were used to identify hapten conjugates that gave optimal sensitivity in immunoassays. The binding and release of PAHs and PAH metabolites from the antibodies was measured to find the best conditions for immunoaffinity separations.
- c) Computational mapping of electrostatic charge distributions proved essential for understanding interaction of the antibodies with bound PAHs and PAH haptens. These experiments provided the first demonstration we know of, for how a relatively neutral spacer arm can change the charge distribution of a hapten, and thus alter its properties as an immunogen and/or a competitor in immunoassay.
- d) Computationally intensive modeling and ligand docking revealed the role of π -cation interactions in PAH binding, furnished estimates of the binding energies, and allowed direct visualization of how PAH symmetry causes cross-reactivity.
- e) Analysis of germline gene usage provided critical information about the genetic origins of the PAH antibodies, and explained why certain combinatorial library architectures were, or were not suitable for deriving functional PAH antibodies.
- f) The extraction method developed by Dr. Li's lab, using supercritical CO₂ with an added chelator, made it possible to concurrently extract the apolar PAHs and their polar metabolites from water, soils, sediments, and coral in a form that could be analyzed directly by instrumental methods and ELISAs.
- g) The immunoaffinity chromatography (IAC) procedures developed by Dr. Li's group provided one-step removal of substances that interfere with PAH and PAH metabolite identification in instrumental analyses.

In summary, this project was a systematic approach, as opposed to the piecemeal way most immunoassay development efforts were made in the past. All of the haptens that might be needed were synthesized by methods that could be used to make additional haptens if necessary. From the start, our work was guided by results from the computational models and docking simulations. In addition, recombinant antibodies and display libraries offer the advantages of enormous repertoires and the ability to engineer changes. Although we were unable to achieve some of our objectives, our work demonstrated how antibody engineering and phage

display can produce desired antibodies unlikely to be obtained by traditional hapten synthesis, immunization, and hybridoma methods.

RESEARCH OBJECTIVES

As noted in the Executive Summary, this project was conducted to remove the major barrier to the timely development and use of more versatile antibody-based detection and sample cleanup methods. The main objective was to adapt combinatorial antibody library and antibody engineering methods for preparing a panel of antibodies to detect different analogs of a toxic substance that is a serious problem for DOE's remediation, regulatory monitoring, ecotoxicology, and human health effects missions. PAHs and their metabolites and adducts are a particularly important and challenging test case. Their unique properties make it impractical to generate antibodies with the required diversity, specificity, and selectivity by older immunological techniques. We sought to develop a knowledge base for how the parent compounds, haptens, and various analogs are bound, and to use that information to design and modify recombinant antibodies, so that inherent cross-reactivity can be interpreted by pattern recognition methods. A secondary objective was to devise sample extraction methods that efficiently and reproducibly recover PAHs and PAH metabolites in a solvent system compatible with instrumental analysis as well as immunoassay, while reducing or eliminating substances that interfere with these methods. In addition, we wanted our work to serve as an example for how multi-analyte assays for other large classes of toxic pollutants such as PCBs could be produced and brought into practice with substantially less cost, labor, and development time.

METHODS

PAH targets for this project were selected to meet six criteria: (a) Designation as priority pollutants, (b) abundance and frequency of occurrence as a problem for DOE, (c) properties consistent with practical use of immunoassay and immunoaffinity techniques, i.e., comparatively high aqueous solubility (0.002-30 $\mu\text{g}/\text{mL}$) and vapor pressures of 10^{-7} - 10^{-2} Torr at 20 $^{\circ}\text{C}$), (d) documented evidence that the parent compound or primary metabolite causes adverse human and ecological health effects (acute toxicity, carcinogenicity, endocrine disruption) or is a biomarker (for example, 1- and 2-aminonaphthalene, 1-hydroxypyrene, and 1-hydroxy- and 6-nitrochrysene), (e) commercial availability of reference materials and precursors for hapten synthesis, and (f) feasibility of attaching aliphatic spacers, preferably at more than one position, so that the PAH moiety could be presented in more than one orientation.

The PAH haptens synthesized for this project are shown in **Figure 1**. All were prepared using either a Friedel-Crafts or a Wittig reaction¹ that could also be used to synthesize other substituted PAH haptens. The haptens were covalently coupled to proteins, and the conjugates were used for immunoassays and selection of display phage, as published.^{1,2}

Immunoaffinity chromatography methods: PAH immunoaffinity columns were prepared using affinity-purified MAb 4D5 IgG, and used as described.³

Computational chemistry and structure modeling: X-ray crystallographic data for PAHs were obtained from the Cambridge Structural Database Centre.^{4,5} The methods for modeling the conformations, van der Waals surfaces, and electrostatic charge distributions of PAHs and PAH haptens using the programs Gaussian94⁶ and DelPhi⁷ have been published.^{2,8} Three-dimensional structures of the antibody binding sites for benzo[*a*]pyrene

and other PAHs were modeled by homology with high-resolution crystallographic structures in the Antibody Structure Database (ASD) developed by Roberts et al.⁹ Geometric accuracy of the models was analyzed using the program PROCHECK.¹⁰ Energy minimizations were done with the program X-PLOR¹¹ using the all-atom force field CHARMM2. Computational docking of key PAHs and haptens into the modeled antibody binding sites was done with the program AUTODOCK.¹²⁻¹⁴ All operations were visualized in INSIGHT II (Molecular Simulations, Inc., San Diego CA) on Silicon Graphics workstations.

Antibody engineering and selection by phage display: The PAH-binding hybridoma lines 4D5 and 10C10, from which we cloned the Fab domains, were originally derived¹⁵ and generously provided by Prof. Regina Santella (Cancer Research Institute, Columbia University, New York NY). The pComb3 and pComb3H phagemid vectors were provided by Dr. Carlos Barbas (The Scripps Research Institute, La Jolla CA). Initial clones were prepared in pComb3^{16,17} with funding from the Office of Naval Research. During the DOE EMSP grant, these rFabs were further characterized, and new clones were prepared in pComb3H.¹⁸ All procedures were as we previously described for rFabs to the herbicide diuron¹⁹ and rFabs to coplanar polychlorinated biphenyls.²⁰ Site-directed mutagenesis was done with a QuikChange site-directed mutagenesis kit (Stratagene, Inc., San Diego CA) according to the manufacturer's instructions.

The "Nissim" and "Fab2 LOX" large combinatorial phage display libraries for human single-chain Fv antibodies (scFvs) and rFabs, respectively, were obtained from Prof. Greg Winter's laboratory (Medical Research Council, Cambridge, UK).^{21,22} Display phage were subjected to several rounds of selection and enrichment using paramagnetic beads coated with various PAH haptens conjugated to bovine serum albumin (BSA) or cytochrome c, according to protocols supplied with the libraries and described in our previous work.^{19,20}

Enzyme immunoassays (ELISAs) to identify phage-displayed and soluble scFvs and rFabs were performed as previously described.^{19,20} Affinities for various PAHs and PAH haptens were determined by quenching of antibody fluorescence,²³ ELISA²⁴, and/or kinetic exclusion fluoroimmunoassay (KinExA).²⁵⁻²⁸

PAH residue recovery and analysis methods: New protocols were developed for cleanup of PAHs and the metabolite 1-hydroxypyrene from surface water, marine sediments, and coral prior to instrumental analysis. Initial extracts were made in supercritical CO₂ containing 5% (w/w) EDTA and 50% (v/w) methanol using an SFX 2-10 instrument (Isco, Inc., Lincoln, NE), as previously described.²⁹ For immunoaffinity chromatography, affinity-purified MAb 4D5 IgG was covalently attached to beads made of hydrophilic bisacrylamide-azlactone copolymer (Ultralink, Pierce Chemicals, Rockford IL). The beads were packed in 1 x 11 cm polypropylene columns and used as published.³

RESULTS

Development of new PAH haptens. Haptens are target compounds or their mimics, with a spacer arm that can be linked covalently to proteins and solid-phase materials. Haptens are essential for evoking desired antibodies by immunization, for selecting recombinant antibodies from phage display and other libraries, and as competitors in various types of immunoassays. The number of different PAH haptens that can be made is limited by the symmetry and reactive groups. Multiple haptens are a great asset for antibody and assay development (for example, see Eck et al.³⁰, Székács et al.³¹, and Brandon et al.³²). However, no such set had been reported for PAHs. Our first priority in this project was to synthesize several PAH haptens that could be used to select different antibodies from phage display libraries, and to cause distinctly different cross-reactivities in competition immunoassays.

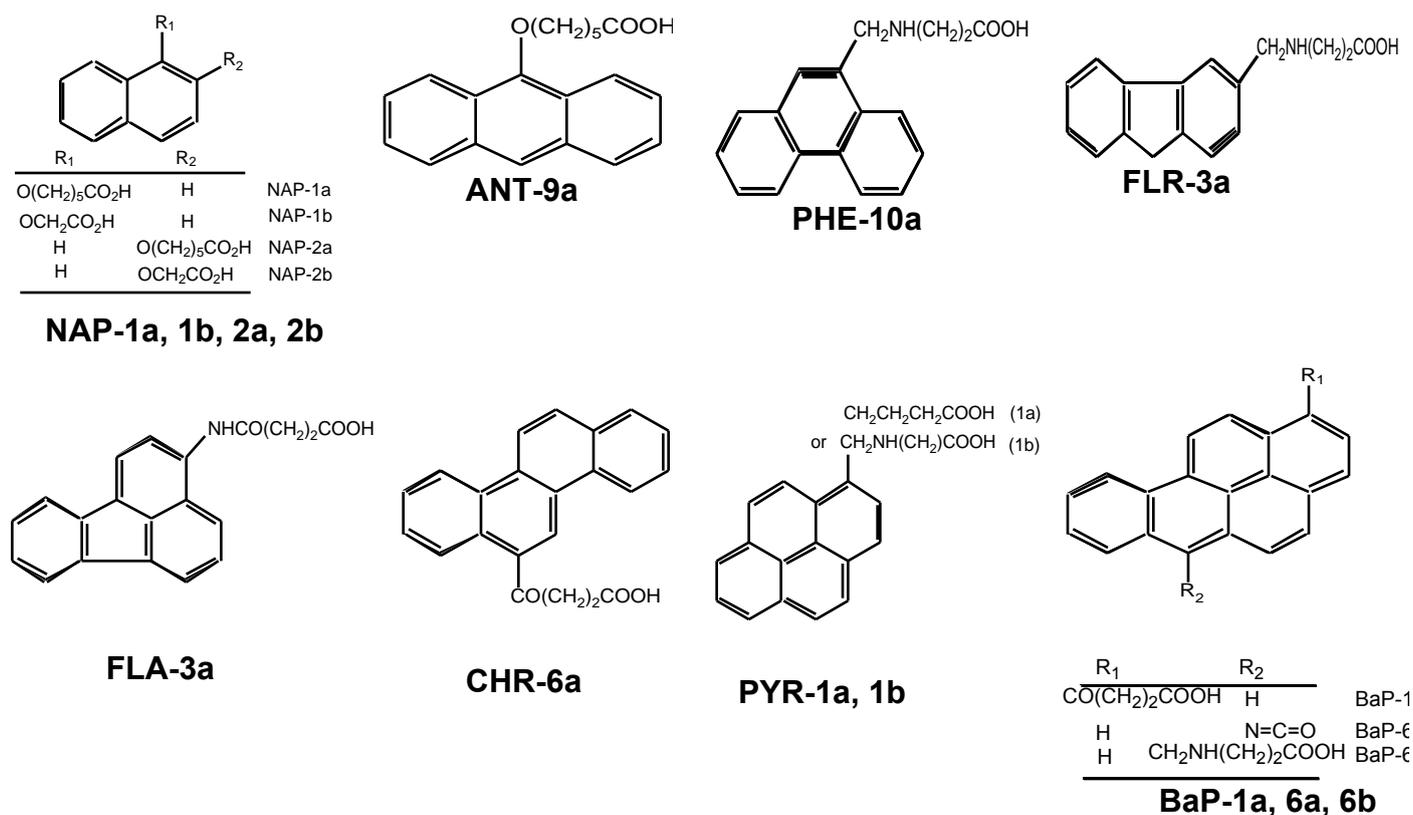


Figure 1. PAH haptens synthesized for this project. PAHs are naphthalene (NAP), anthracene (ANT), phenanthrene (PHE), fluorene (FLR), fluoranthene (FLA), chrysene (CHR), pyrene (PYR), and benzo[a]pyrene (BaP). Numbers refer to the PAH carbon atom that was derivatized.

Figure 1 shows the library of 2-, 3-, 4-, and 5-ring PAH haptens that we synthesized, purified, and verified. All of the haptens were made using one of two general synthesis schemes — a Friedel-Crafts acylation and a Wittig reaction. Most were prepared starting with commercially available precursors.¹ Additional molecular diversity was created by attaching spacer arms of different lengths.

PAH immunoassay performance using the new haptens. Indirect competition ELISAs were done in wells coated with conjugates of the new haptens. Like the original MAbs, the 4D5 and 10C10 rFabs cross-reacted differently with various PAHs, and the cross-reactivity was broader than previously described.¹ (**Figure 2**).

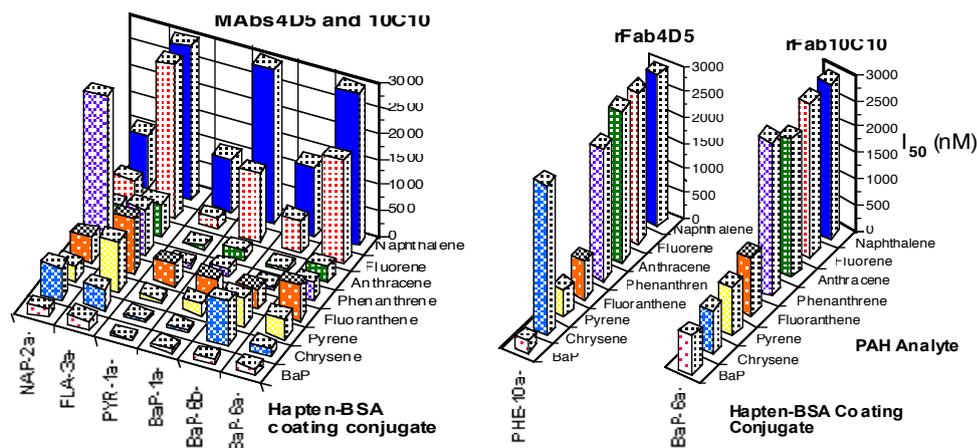


Figure 2. Relative cross-reactivity of MABs and rFabs 4D5 and 10C10 with PAHs in indirect competition ELISAs, using various haptens as competing conjugates. Concentrations (nmoles/L) of PAH analyte required for half-maximal inhibition (I_{50}) are shown. The shorter the bar, the more sensitive the assay.

A simple but previously unpublished method was devised to compare the sensitivity of these assays. Different amounts of a PAH-BSA conjugate were mixed with a limiting amount of antibody in wells coated with BaP-6a-BSA, and half-maximal inhibition of antibody bound to the immobilized conjugate was measured. A second set of competition assays was done with soluble BaP in wells coated with each of the hapten-BSA conjugates. The results of both assays were expressed as a 'recognition index,' defined as a ratio of I_{50} values (**Table 1**). These data showed that the most sensitive ELISAs for soluble PAHs were obtained when the most weakly binding PAH hapten conjugate was used to coat the wells. For example, the most sensitive assay for BaP was obtained using pyrene-1-BSA as the coating conjugate.¹ This test should be useful for optimizing ELISAs for any analyte when two or more hapten conjugates are available.

Table 1. Sensitivity of indirect ELISA for BaP and relative binding of MAb 10C10 to PAH-BSA conjugates.

PAH-BSA conjugate	recognition index and (I_{50} , ppb) ^a	I_{50} (ppb) for BaP with indicated coating conjugate ^b
PYR-1a-BSA	0.55 (51.6)	11.7 (46.8 nM)
PYR-1b-BSA	0.61 (47.0)	28.7
FLA-3a-BSA	0.70 (40.5)	47.2
CHR-6a-BSA	0.75 (38.0)	17.9
ANT-9a-BSA	0.95 (29.9)	14.3
BaP-6a-BSA	1.0 (28.5)	25.5
BaP-6b-BSA	1.2 (23.7)	35.0
BaP-1a-BSA	1.9 (14.9)	14.5
PHE-10a-BSA	2.1 (13.4)	23.4
FLR-3a-BSA	9.2 (3.1)	24.7
NAP-2a-BSA	95 (0.3)	134.0 (536 nM)

^{a,b} All values were the means of data from four replicate wells.

^a An I_{50} value is the concentration of the indicated hapten, in the form of a BSA conjugate, that gives half-maximal inhibition of MAb 10C10's binding to wells coated with BaP-6a-BSA. A limiting amount of MAb is used. Recognition index is defined as the ratio of I_{50} values when soluble BaP-6a-BSA is the competitor, divided by I_{50} when the indicated conjugate is competitor on wells coated with BaP-6a-BSA (2.0 ng/100 μ L/well).¹

^b Indirect ELISAs using MAb 10C10 on plates coated with the hapten-BSA conjugate in the left column.

The results in **Figure 2** and **Table 1** demonstrate why an antibody-based system for identifying PAHs is so difficult to design. In many small-molecule immunoassays, cross-reactivity can be modulated to some extent

by using different haptens as competitors.³³⁻³⁸ The relative binding patterns for BaP and other PAHs differed somewhat when different hapten conjugates were used. However, significant structural overlap blurred the differences in PAH size.

Structural and chemical bases of PAH recognition. Our most important specific aim was to elucidate the molecular interactions that occur when PAHs are bound by the antibodies. The DNA sequences of the rFabs were determined, translated into the amino acid sequences, and used as templates to build and verify models of the 3-dimensional structures of the 4D5 and 10C10 binding sites. The models enabled us to identify the amino acids that form the binding site and interact with PAHs and haptens. At the same time, structures and electrostatic charge distributions of the PAHs and haptens were calculated, based on available small-molecule crystallographic data. Computational docking programs were then used to estimate the steric and energetic requirements for binding. The most important findings, summarized below, provided important insights for engineering improved PAH antibodies.^{2,8}

The role of symmetry was most evident from ELISAs showing that the MAbs and rFabs bound BaP-1a-BSA approximately as well as the BaP-6a-BSA hapten.¹ Since the spacer arm has to protrude from the binding site, this indicated that BaP may be bound in both "right-side-up" and "upside-down" orientations. Computational docking simulations confirmed that both binding modes were energetically and sterically similar, consistent with the ELISA results (**Figure 3A**). To our knowledge, this orientational cross-reactivity has not been described previously. It definitely must be considered in the design of multi-analyte PAH assays, and it probably occurs with other ligand-antibody combinations. **Figure 3B** is a cut-away view of the solvent-accessible surface of the binding pocket of MAb 4D5, with the BaP-6a and BaP-1a haptens bound in the best-scored orientations calculated by AUTODOCK. There was excellent shape complementarity between the haptens and the binding site. The computational methods used and evidence for the model's correctness have been published.^{2,8}

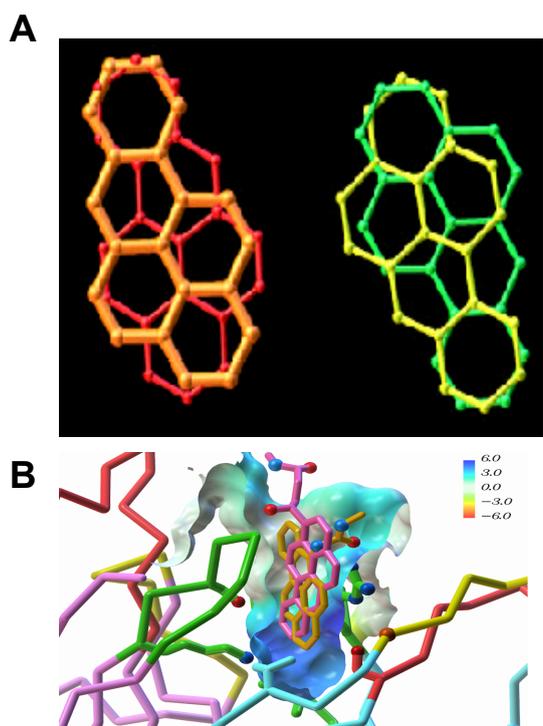


Figure 3. Symmetry of benzo[a]pyrene (BaP) binding revealed by the AUTODOCK simulations. **A.** Superposition of the four most likely orientations for BaP bound in an energy-minimized model of the 4D5 binding pocket. When the domains at the bottom are deepest in the binding site, the topmost parts are slightly exposed to solvent. Colors indicate the free energy, from lowest (most favorable) to highest (least favorable): red < orange < yellow < green. All four orientations are energetically feasible. Thickness represents the number of times each orientation was obtained during 50 docking runs. The orange cluster was the most frequent result, and thus the likely to occur.

B. Solvent-accessible (Connolly) surface of the binding site in the energy-minimized model of 4D5. The computational docking results for the BaP-6a (gold), and BaP-1a (pink) haptens, with the linkers exposed to solvent, were virtually superimposable. BaP-6a docks with the pyrene moiety deepest in the pocket, as with the red and orange orientations in panel A. The BaP 1a hapten docks with the pyrene moiety upward, corresponding to the yellow and green orientations in panel A. Thus, the pocket can accommodate both orientations of BaP, as well as other PAHs.

The modeling and docking studies indicated that the side chains of lysine L89 and arginine H95 lay on opposite sides of the binding pockets, suggesting that PAH binding was due at least partly to interaction with a positive charge imparted by these side chains. To test this hypothesis, mutations were introduced to replace these side chains with ones that had neutral charge. Lysine L89 was replaced with methionine (K → M), and arginine H95 was replaced by glutamine (R → Q). The original rFab 4D5 and the L89 K → M mutant bound all of the PAH haptens about equally well (**Figure 4**). In competition ELISAs, the L89 mutant of 4D5 competitively bound soluble BaP, pyrene, and fluoranthene with half-maximal inhibition (I_{50}) values comparable to those with wild-type 4D5. Unlike the wild-type 4D5, however, the L89 mutant did not *competitively* bind phenanthrene, anthracene, fluorene, or chrysene. Calculations indicated that lysine L89 has an unusually low pKa, and is probably uncharged due to its hydrophobic environment.⁸ By contrast, variants with the H95 R → Q mutation or the double mutation showed no detectable binding of BaP and PAH haptens. Thus, the positively charged side chain of arginine H95 is essential for PAH binding, while lysine L89 has negligible, or very subtle effects on PAHs recognition.

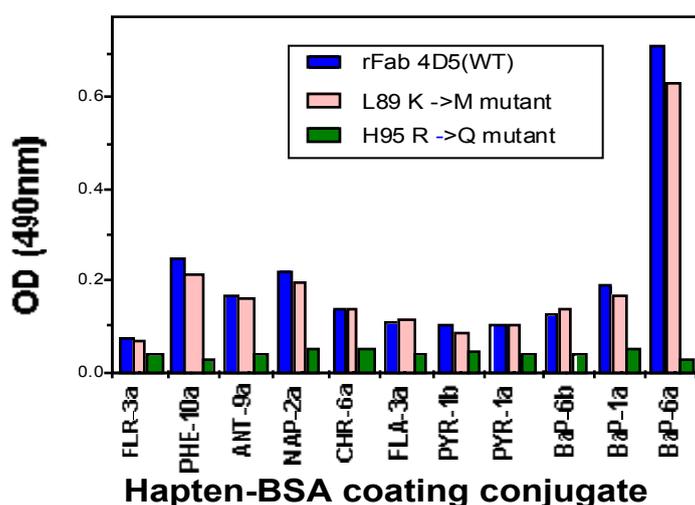


Figure 4. Performance of rFab 4D5 variants in which Lys L89 and Arg H95 were replaced by amino acids with neutral side chains. Binding was measured by indirect ELISAs on wells coated with the indicated PAH hapten-BSA conjugates in Fig. 1. PAHs are fluorene (FLR), phenanthrene (PHE), anthracene (ANT), naphthalene (NAP), chrysene (CHR), fluoranthene (FLA), pyrene (PYR), and benzo[a]pyrene (BaP).

The 4D5 and 10C10 antibodies had three distinctive unusual features. First, the PAH-binding pockets were exceptionally deep — about 17 Å from the C atom of valine H47 to the C atom of tyrosine H97. The depth was due to replacement of tryptophan at the highly conserved framework position H47 by isoleucine in 4D5 and by valine in 10C10. Without this substitution, the antibodies could not have accommodated BaP or similar large PAHs. The widest span in the pockets was about 7 Å, from the C atom of proline L95 to the C atom of arginine H95.²

Second, although the sequences of rFabs 4D5 and 10C10 differed by 23 amino acids, the binding pockets are very similar. Only three side chain differences occurred in the binding pockets. Of the 20 other amino acid differences in 4D5 and 10C10, nine were in portions of CDRs L1 and H2 that did not contact BaP, and the rest were at various sites on the antibody surface that were not likely to affect PAH binding (**Figure 5**). Although only 11 of the substitutions were structurally conserved (for example, threonine to serine and phenylalanine to tyrosine), 4D5 and 10C10 performed similarly in ELISAs, and their equilibrium dissociation constants (K_d) for BaP were similar. In addition, independently prepared computational models of the binding pockets were virtually the same, and docking simulations showed only a minor shift of BaP docked in the pockets.

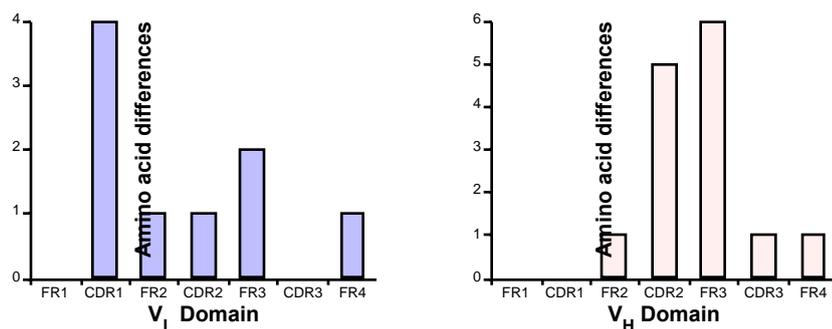


Figure 5. Distribution of amino acid differences in framework (FR) and complementarity-determining regions (CDRs) of the V_L (left), and V_H (center) domains of rFabs 4D5 and 10C10.³⁹

Third, analysis of germline gene usage indicated that 4D5 and 10C10 resulted from a rare and stringent biological selection. Both antibodies arose from the same mouse germline V-region genes, V_L 8-21 and V_H 7183.10, which are expressed very seldom in B cells. V_H 7183.10 encodes a leucine at position H47, as found in 10C10. 4D5 has valine at H47, evidently acquired by somatic mutation. In addition, arginine H95, the first residue of CDR H3 in both antibodies, was created by a two-nucleotide addition (GG) during recombination at the junction between V_H 7183.10 and the germline D-region sequence DSP2.2.²

Figure 6 is a visual summary of the PAH binding site topology and putative charge interactions with BaP. The molecular models and assay results revealed unusual structural characteristics of 4D5 and 10C10, providing specific guidance for creating libraries of new variants that could be useful for discriminating among PAHs. In many antibodies, a change of one amino acid can abolish ligand binding, even if the changed residue is not part of the binding pocket.^{40,41} This was true for arginine H95, and (by inference from the models) tryptophan H47 in 4D5 and 10C10. On the other hand, these antibodies have remarkably similar structural and performance attributes despite the other amino acid differences.

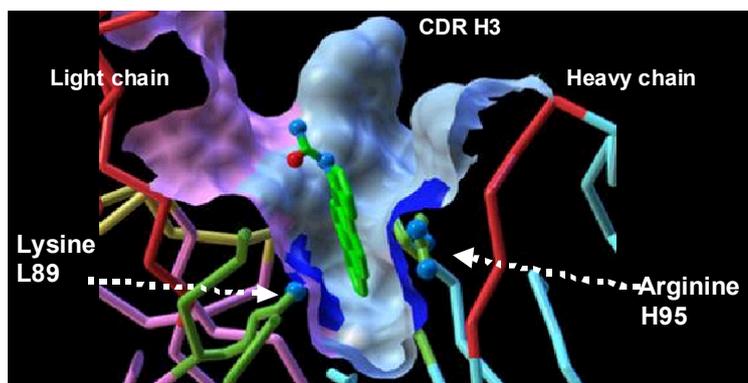


Figure 6. Solvent-accessible surface of rFab 4D5, with BaP-6a hapten positioned by the computational best fit in the binding site. The portion of the surface contributed by the light chain variable region is magenta. The light blue portion is the surface contributed by the heavy chain. Dark blue patches denote regions of positive charge contributed by the side chain amino group of Lys L89 (green with blue N atom) on the left and the guanidinium group of Arg H95 on the heavy chain (green with two blue N atoms) on the right. The large bulge in the surface (top, center) is the top of the CDR H3 loop, which does not play a major role in forming the binding pocket.

Lessons learned from use of very large human antibody phage display libraries. At the time we began this project, our laboratory had experience attempting to derive rFabs specific for the herbicide diuron from one very large combinatorial human rFab phage display library developed by Barbas et al. This library was produced by combinatorial mutagenesis of CDRs in an antibody to tetanus toxoid.^{18,42-44} We recovered antibodies that bound chlorophenolic haptens, but none competitively bound the parent compound (the herbicide diuron) in solution.⁴⁵ In the present project, we first attempted to select rFabs that bound benzo[*a*]pyrene or fluoranthene haptens from the Fab 2LOX library described in Methods. The unsuccessful

outcome was summarized in our Year 1 progress report, and in the extended abstract for the July 1998 EMSP Workshop.⁴⁶

We selected and enriched hapten-specific binders about 10^6 -fold from the FAB 2LOX library. However, analysis by PCR revealed that the isolates had only heavy (H) chains. We repeated that test on the original library, and found that only about 10% of the sequences had both light (L) and H chains. The library did not include vectors that only had L chains. The library's developer, Dr. Andrew Griffiths, confirmed our observations. Although this had negligible effect on the total diversity (10^{11} instead of 10^{12} members), it greatly reduced the efficiency of selection. To correct this, we first isolated and amplified phage that had L, as well as H chains, by capture on magnetic beads coated with goat anti-human L chain antibodies. This population was panned in microwells to remove non-specific hydrophobic binders,⁴⁷ and then panned to remove biotin-binding display phage that might interfere with the detection step in subsequent phage ELISAs. When the remaining phage produced no binders specific for BaP or fluoranthene haptens, we discontinued work with this library.

A similar attempt was made to isolate PAH-binding antibodies from the Nissim single-chain Fv (scFv) library. This library was comprised of roughly 10^8 human V_H sequences, all of which were linked to a single human V_L sequence from an antibody to bovine serum albumin (BSA). The library phage were first panned to remove antibodies that bound plastic and biotin. To eliminate antibodies that bind to a carrier protein, the remaining phage were alternately panned on BSA and cytochrome *c* conjugates of each hapten. We recovered six scFvs that bound naphthalene-2a- hapten and six that bound phenanthrene-10a hapten. Only one scFv, designated Nap-2, competitively bound soluble naphthalene, with I_{50} values of 150-600 ppb. In another unexpected instance of PAH cross-reactivity, Nap-2 scFv also bound pyrene, which is structurally equivalent to two fused naphthalenes.

Although the Nissim library reportedly yielded scFvs that bound fluorescein, 4-hydroxy-5-iodo-3-nitrophenylacetyl, and 2-phenyl-5-oxazolone haptens,²¹ its unusual construction is not well suited for deriving antibodies to small organic molecules. The V_L domain common to all Nissim scFvs came from an antibody to an epitope on bovine serum albumin. All of the diversity is in the V_H domain, which is often the predominant ligand-binding element. Nissim antibodies had only one linker sequence, which may not be optimal for proper folding and formation of the V_H - V_L interface. Furthermore, the odd architecture of the Nissim antibodies makes homology modeling and model validation difficult and less reliable. Most of the sequence diversity is in CDR H3, which is the most flexible and difficult region to model. Despite these shortcomings, we decided to make a computational model of Nap-2 and learn what we could from it.

The homology model showed that the human Nap-2 scFv had a shallower binding pocket than mouse rFabs 4D5 and 10C10. However, Nap-2 also had two arginine side chains, one on each side of the binding site. Thus, it is possible that this scFv may use a π -cation interaction to bind PAHs.² In addition, our ELISA results were corroborated by docking simulations, which indicated that Nap-2 could bind pyrene in approximately the same orientation as it was bound in the murine rFabs 4D5 and 10C10 (Pellequer et al., in preparation).

In summary, our extensive efforts with the phage display libraries produced no antibodies with improved performance for practical use. However, the results revealed principles for successful design and use of antibody libraries for PAHs and other small molecules in the future. We stated the most important of these in one of our most recent publications:²

One conclusion from this study underscores the importance of understanding binding-site architecture for the design and use of combinatorial antibody phage display libraries. It is not sufficient to assume, based on

probability alone, that alternative binding motifs for almost any analyte may be found in very large, diverse libraries. Other essential factors include binding-site differences for epitopes on proteins (cup-like), peptides (channel-like), and small haptens (deep cleft),^{48,49} the possibility of a conformational change upon antigen binding,⁵⁰ genetically conserved preferences among CDR loops,⁵¹ and differences in structure and expression of V genes from the murine and human repertoires.^{52,53} The latter suggests why the Fab 2LOX and Nissim libraries were not likely to yield desirable antibodies for PAHs. Virtually all known human V_H genes have a Trp, and none have Val or Leu, at position H47. Consequently, the human V gene germline repertoire may not include sequences that can form the unusually deep binding pocket required to bind BaP. By contrast, a library made by mutagenesis of 4D5 or 10C10 should be a good source of useful variants.

Method development for PAH residue recovery and analysis: This portion of the project was conducted exclusively by Dr. Li's group. It focused on methods to make practical use of the antibodies, and on pilot studies to assess accuracy, reliability, and other performance properties. Specific aims were (a) to develop or modify conventional methods for recovering parent PAHs and PAH metabolites in a form compatible with immunoassay as well as instrumental analysis, (b) to adapt immunoaffinity methods for robust, efficient recovery of PAHs from environmental and biological samples, and (c) to conduct small-scale pilot studies relevant to DOE needs, to demonstrate the practicality of methods from aims a and b, and to identify and solve problems that arise. The major accomplishments and results are summarized as follows:

Aim (a): Two key problems were addressed: recovery of apolar parent PAHs and their polar metabolites in one step, and keeping the recovered residues soluble in a form that allows analysis by ELISA (an aqueous system), gas chromatography-mass spectrometry (GC-MS), and other instrumental methods that require organic solvent.

1. A supercritical fluid extraction (SFE) method using CO₂ supplemented with Na₄EDTA was developed for recovering apolar parent PAHs, 1-hydroxypyrene (1-pyrenol), and more than 20 other polar and apolar pesticides and herbicides from soil, with efficiencies of 85-104% (**Table 2**).^{29,54} Extracts prepared with this technique could be used in competition ELISAs and GC-MS.
2. Pressurized fluid extraction (PFE) procedures, developed and tested for analysis of polar pesticides and herbicides, were adapted for recovery of PAH metabolites. These methods are much faster and use less organic solvent than conventional Soxhlet procedures.^{55,56}
3. Twelve PAHs and 20 PCBs were recovered from marine sediments, fish, and coral, with efficiencies of 64-112%, using the SFE and PFE protocols described above, with minor variations.
4. A high pressure liquid chromatography (HPLC) method was developed for analysis of 1-hydroxypyrene (1-pyrenol), a human urinary biomarker of PAH exposure. The levels of urinary 1-hydroxypyrene correlated strongly with cigarette use.⁵⁷

Table 2. Recoveries of PAHs and PAH metabolites from soil using SFE^a

Analytes	Recovery ^a ± SD ^b , %	
	no Na ₄ EDTA	with Na ₄ EDTA
PAHs (spike level, 10 µg/g)		
naphthalene	95 ± 5	93 ± 8
phenanthrene	100 ± 8	99 ± 13
anthracene	94 ± 9	97 ± 9
pyrene	93 ± 12	101 ± 10
benzo[a]pyrene	85 ± 15	95 ± 3
PAH metabolites		
benzoic acid	44 ± 3	100 ± 4
2-naphthoic acid	56 ± 5	100 ± 3
1-naphthylacetic acid	45 ± 3	90 ± 5
1-naphthol	92 ± 6	94 ± 4
9-phenanthrol	63 ± 5	96 ± 2
1-hydroxypyrene	67 ± 7	99 ± 5
Other compounds		
phenol	97 ± 4	96 ± 5
resorcinol	97 ± 4	102 ± 1
pentachlorophenol	97 ± 6	104 ± 2

^a Data excerpted from Guo et al., 1999, *Anal. Chem.* 71:1309-1315. Spike levels were 0.01-10 µg/g soil.

^b SD = standard deviation of the mean

Aim (b): This task addressed the problem that SFE and PFE extracts of marine sediments, coral, and other matrices, contained too many other substances that interfered with direct analysis of PAHs, PAH metabolites, and adducts. Immunoaffinity methods were developed to provide the necessary cleanup.

1. Immunoaffinity columns were prepared from affinity-purified 4D5 and 10C10 MAb and rFab IgGs. Experiments were performed to determine the range of pH, ionic strength, and organic solvent types and concentrations that allowed BaP and other PAHs and metabolites to be bound or released. The data were presented in Li, et al.³
2. The optimized operating conditions were used to automate cleanup of the PAH analytes in SFE extracts of pristine marine coral spiked with BaP. The method proved to be efficient, and the column could be regenerated and re-used at least twice (**Figure 7**).

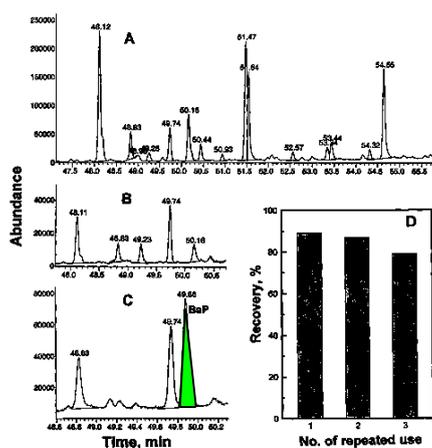


Figure 7. Immunoaffinity cleanup of SFE from marine coral samples spiked with BaP. Shown are GC-MS total ion chromatograms of the applied SFGE extract (A), column wash (B), and eluted fractions (C) from an immunoaffinity column made with MAb 4D5. All of the BaP remained on the column until the elution step. In this experiment, BaP eluted at 49.88 min. The unidentified peak immediately to the left of BaP (49.74 min) was partially removed in the wash step (B), but some co-eluted with BaP (C). The spike recovery remained efficient through three consecutive regenerations of the affinity column (D).

Figure 8 shows the extent of the cleanup and resolution of BaP in SFE preparations of coral. Immunoaffinity chromatography with MAb and rFab 4D5 removed over 90% of the interferences from supercritical fluid extracts from corals.

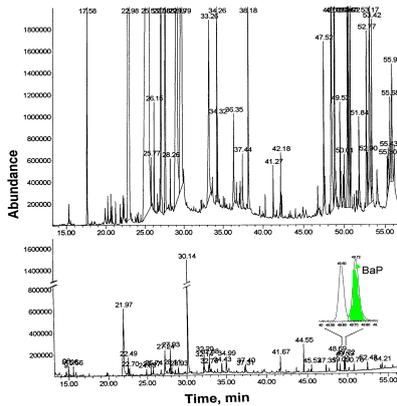


Figure 8. GC-MS total ion chromatograms of a CO₂ supercritical fluid extract (SFE) of coral, before (*upper panel*) and after (*lower panel*) cleanup on an immunoaffinity column made with MAb 4D5. Benzo[*a*]pyrene (BaP) eluted at 49.72 min, and was almost entirely separated from the adjoining peaks, including the one immediately to its left (49.60 min), which were not identified in this experiment.

Aim (c): The methods developed in Aims (a) and (b) were tested in small-scale demonstration projects of several types. Correlations between ELISA and GC-MS or HPLC data were determined in extracts from surface water, soil, sediments, fish, crabs, and corals.

containing naturally incurred and spiked BaP and other PAHs. Through a collaboration with biologists in the U.S. Fish and Wildlife service, samples were compared from sites known to be polluted or relatively relatively pristine. Particular attention was given to collection of baseline data and identification of substances that interfered with or biased the assays.

1. Indirect competition ELISAs were used to compare recovery of BP spikes (0.5-151 ppb; 2-600 nM) in phosphate buffer, tap water, unfiltered water from the Ala Wai canal in Honolulu, and urine. Recoveries averaged $88 \pm 6\%$ to $95 \pm 13\%$ in buffer and water, and $111 \pm 17\%$ in urine. No significant matrix effects were observed with these samples, but the ELISA results were uniformly higher than GC-MS results with urine samples.³ ELISA and GC results with these samples correlated very closely (**Figure 9**).

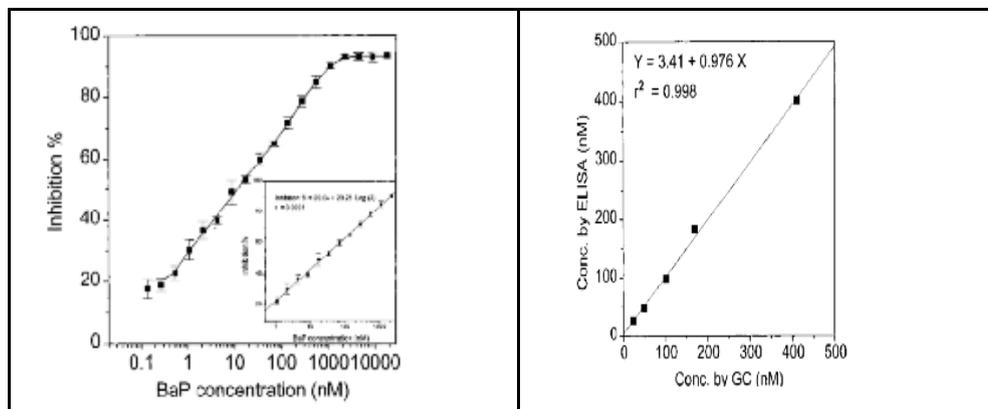


Figure 9. (*Left panel*) Dose-response of indirect competition ELISA for BaP with MAb 10C10, using BaP-6a-BSA as the coating conjugate. (*Right panel*) Correlation between ELISA and gas chromatography for samples of canal water spiked with different amounts of BaP.

2. A limited study was done on PAH bioaccumulation of in marine coral. In addition to its great importance as the basis for an entire marine ecosystem, coral's mineralized exterior, living tissue, and associated symbiotic bacteria. make it an exceptionally complex material for analysis. A SFE procedure was adapted for PAH recovery from *Porites compressa*, a non-endangered species. Samples were obtained by the U.S. Fish and Wildlife Service from Trig Island, a relatively pristine reserve in the French Frigate Shoals northwest of Hawaii, and from Kaneohe Bay, a significantly polluted area on Oahu. Nine PAHs were identified by GC-MS, and all except fluorene were present at significantly higher levels in the samples from Kaneohe Bay (**Table 3**). PAHs added as pre-extraction spikes were recovered with efficiencies of 85-108%, determined by GC-FID and GC-MS.⁵⁸

Table 3. PAHs found in *Porites* coral by GC-MS.

PAH	Concentration (ng/g)	
	Kaneohe Bay	Trig Island
Fluorene	ND	7
Pyrene	25	9
Fluoranthene	26	9
Anthracene	21	4
Phenanthrene	26	15
Chrysene	40	ND
Triphenylene	24	ND
Benzo[a]pyrene	45	ND
Benzo[e]pyrene	37	ND

3. Immunoaffinity columns prepared with MAb 4D5 or 10C10 gave 58-73% recovery of BaP standards in phosphate buffer, but only about 40% recoveries from coral. The columns could be re-used up to seven times. Improvement of PAH recovery from coral extracts had to be suspended until a new stock of the purified MAb could be made.
4. ELISA and GC-MS results were also compared for measurement of PAHs, and 1-hydroxypyrene as a biomarker, in surface water and sediment collected from the Pearl Harbor and James Campbell National Wildlife Refuges (NWRs) in Hawaii. Water samples were extracted by solid phase extraction (SPE) with C₁₈ resin, while sediments were extracted by SFE as described above. Na₄EDTA was more effective than Na₂SiO₃ as an SFE additive for 1-hydroxypyrene recovery from sediments. The PAH concentrations, determined by ELISA and expressed as benzo[a]pyrene equivalents, were consistently higher than those obtained by GC-MS (**Table 4**). We did not determine whether this was due to substances that inhibited the ELISA to some extent, or simply the fact that ELISA responds to all cross-reactive substances that are present, while GC-MS quantifies individual compounds.

Table 4. Comparison of ELISA and GC-MS estimates of PAH concentrations in sediments from National Wildlife Refuges in Hawaii, recovered by SFE.⁵⁹

Sediment sample	Na ₄ EDTA added in SFE	Estimated PAH concentration (ng g ⁻¹)	
		ELISA ^a (BaP equivalents)	GC-MS
A	+	502 ± 74	356
A	-	365 ± 69	282
B	+	345 ± 15	209
B	-	259 ± 27	166
C	+	531 ± 63	287
C	-	423 ± 19	199

^a. Data were average of four replicates.

5. In collaboration with Dr. Garry Rechnitz at the University of Hawaii, we developed and tested two prototype immunosensors. A capacitive immunosensor was made by covalently attaching purified 10C10 IgG to a monolayer of cystamine that self-assembled on a gold electrode. Binding of BaP-6a-BSA and pyrene-1a-BSA conjugates at concentrations of 0.01-6.0 μM was detected by linear sweep voltammetry, as a decrease in the charging current.^{60,61} A piezoelectric flow-through immunosensor was also constructed. Pyrene-1a-BSA conjugate was tethered, through a linker of thioctic acid, to the surface of gold coated quartz crystals that had a basic resonant frequency of 10 MHz. This sensor could be used to detect 10C10 antibody, and soluble BaP, pyrene and naphthalene.^{61,62} These experiments demonstrated the potential for incorporating these antibodies in practical PAH sensor devices.

RELEVANCE, IMPACT, AND TECHNOLOGY TRANSFER

1. *How does this new scientific knowledge focus on critical DOE environmental management problems?*

For more than 20 years, DOE programs brought about major advances in electronic and fiber optic sensors, microarrays, and microfluidic systems, and other devices and analytical instruments that use antibodies and other biomolecules to rapidly identify particular hazardous substances. Critical applications include rapid hazard assessment in emergencies, high-throughput, low-cost surveys to define contaminated sites, characterization of mixed wastes, detection of worker exposure biomarkers, evaluation of protective measures, ecological risk analysis, and compliance monitoring before, during, and after remediation. However, deployment and practical use of antibody-based sensing devices has been slowed because there have been too few of the most suitable antibodies, particularly those with subtle but interpretable differences in recognition (cross-reactivity) with members of a large class of similar compounds such as polynuclear aromatic hydrocarbons (PAHs). It is virtually impossible to produce such antibodies by traditional *in vivo* and hybridoma methods.

Our project focused on combined use of knowledge-based molecular structure modeling and *in vitro* antibody engineering as enabling technology to meet DOE's immediate and long-term needs for customized antibodies. PAHs and their metabolites are a concern in many DOE environmental management and health effects problems. The physical and chemical properties of PAHs make them very challenging to extract and identify by any method.

When we proposed this project in 1996, antibody engineering had evolved to a stage where it was generally expected to replace conventional immunization and hybridoma methods in the next few years. While our work was under way, many laboratories worldwide made remarkable progress, and solved many problems that we also encountered. Although regrettably we did not achieve our ultimate goal of producing improved recombinant antibodies for multi-analyte PAH analysis, our work brought that goal much closer.

2. *How will the new scientific knowledge that is generated by this project improve technologies and cleanup approaches to significantly reduce future costs, schedules, and risks, and meet DOE compliance requirements?*

Each part of this project contained approaches and findings that could potentially reduce cleanup costs, timelines, and risks. Our work produced new knowledge in five critical areas:

- a. We determined the submolecular interactions that occur when PAHs are bound by the antibodies we studied. Most importantly, we found that the progenitor binding site for the five-ring carcinogenic PAH, benzo[a]pyrene (BaP), existed in the murine germline V gene repertoire, but not the human repertoire.
- b. We demonstrated the advantages and shortcomings of the two major genetic engineering strategies used to derive improved antibodies: use of very large combinatorial phage display libraries, *versus* successive rounds of *in vitro* mutagenesis and selection from an antibody that was able to bind the ligand.
- c. We used simplified, generalized reaction schemes to create a library of PAH haptens that represent the major PAH priority pollutants that are measurable by immunoassay. The hapten library fills a need that was obvious, but to our knowledge not filled, for more than a decade. These haptens are crucial for selection of new antibodies, metabolite identification, and formatting of immunoassays with different PAH binding patterns.
- d. Supercritical fluid extraction (SFE) and pressurized fluid extraction (PFE) methods were developed for efficient recovery of polar metabolites as well as the apolar parent PAHs. The extracts are compatible with ELISAs and confirmatory analysis by GC-MS and HPLC.
- e. Immunoaffinity chromatography (IAC) methods were developed to recover BaP from a variety of environmental and biological samples. IAC proved to be particularly effective for removing interfering substances from the SFE and PFE extracts.

DOE compliance requirements can be met by using immunochemical methods that satisfy site-specific, cleanup stage-specific, performance-based data quality objectives.⁶³⁻⁷¹ EPA's validation and adoption of immunoassay methods (the 4000- series in SW-846) was, and continues to be, accomplished largely at DOE sites with DOE participation. DOE, EPA, manufacturers of immunoassay systems, and other stakeholders have a vested interest in refining immunochemical test methods to satisfy compliance requirements.

As immunoassay and other new methods evolved and were used successfully over the last several years, a consensus opinion developed that the results from such methods could be accepted for compliance purposes, if they met site-specific, analyte-specific data quality objectives (DQOs). In other words, the analytical results would be considered valid and conclusive if all of the individual procedures could meet the DQOs for the target

analyte in the matrix of concern at the required action level. Rigorous, explicit DQOs now exist for sample extraction, cleanup, assay methods, sampling plans, transport and storage, data evaluation, and all other project elements.⁶³ For particular projects, this “performance-based” approach is much more flexible and economical than the traditional prescriptive requirement that only promulgated methods are acceptable. The definitive EPA policy on use of immunochemical methods in this way, and the misconceptions that have influenced the regulatory community, is spelled out by Lesnik et al.⁷² Performance-based validation will gain increasing importance as next-generation methods based on antibody arrays, multiplex bead immunoassays, and similar multi-analyte, high-throughput technologies are brought into practice.

3. *To what extent does the new scientific knowledge bridge the gap between broad fundamental research that has wide-ranging implications, and the timeliness to meet needs-driven technology development?*

This is answered partly in our response to question (B). In the antibodies we studied, the interaction between π -electrons on the PAH and cationic amino acid side chains in the antibody, were an essential binding mechanism. This provides a basis for an iterative process for deriving new PAH antibody variants. Knowledge-based antibody engineering is broadly applicable to needs for identification of small toxic molecules by antibodies and other recognition molecules such as peptides, receptors, ion channel proteins, and nucleic acid aptamers.

Our work also revealed fundamental structural and genomic reasons that human antibody phage display libraries are not likely to be a source of antibodies to BaP, and possibly other PAHs. Obviously, a great deal of effort and money may be saved by checking that a phage display library includes sequences likely to be compatible with structural features of ligands of interest. The PAH haptens and the new analyte extraction and cleanup procedures we developed will also facilitate development of new PAH detection systems.

4. *What is the project's impact on individuals, laboratories, departments, and institutions? Will results be used? If so, how will they be used, by whom, and when?*

Our findings will help other investigators developing similar needs-driven antibody technologies to avoid or work around the problems we encountered. The materials and procedures we developed are available to all who can make use of them. Published results from our project^{2,8} were supported by Scharnweber et al., who found that BaP binding to a new MAb they developed was sensitive to changes in ionic strength, consistent with the π -cation binding interaction.⁷³ Dr. Pellequer, who now heads his own laboratory in France, plans to follow up on this in a collaborative project with Prof. Dietmar Knopp and co-workers who developed the new antibody in Munich.

Over the past ten years, an increasing number of laboratories worldwide with objectives similar to those in our project have developed capabilities in computational modeling, antibody engineering, and creation and use of large antibody libraries for environmental and health effects research. Examples include Dr. Larry Stanker, Director, Foodborne Contaminants Research Unit, USDA Western Regional Research Lab, Albany, CA; Prof. Michael R.A. Morgan, Director, Institute for Food Research, Norwich, UK; Prof. Brent Iverson, Dept. of Biochemistry, University of Texas, Austin TX; Prof. J. Christopher Hall, Dept. of Environmental Biology, Univ. of Guelph, Ontario, Canada; Drs. John Skerritt and Amanda Hill, CSIRO Division of Plant Industry, Acton, Australia; and Prof. Bruce Hammock, Dept. of Entomology, Univ. of California, Davis, CA. This trend appears certain to continue. Future projects of this type will be done by multidisciplinary teams. Various parts of the work will require expertise from computational, analytical, bio-organic, and materials chemists, molecular, structural, and environmental biologists, toxicologists, and persons skilled in scaled-up *in vitro* expression of recombinant proteins, high-throughput screening method development, preparation and use of

microarrays, etc. Individuals with these talents may be found in most universities, the DOE National Laboratories, research institutes such as Scripps, and private companies such as Strategic Diagnostics (Wilmington, DE).

5. *Are larger scale trials warranted? What difference has the project made? Now that the project is complete, what new capacity, equipment, or expertise has been developed?*

The SFE and PFE methods for PAH extraction and the IAC method for cleanup just before instrumental analysis should be modified according to newer information published after we completed our work. In particular, IAC should be tested using rFabs encapsulated in a sol-gel glass (SGG) matrix.⁷⁴⁻⁷⁸ When antibodies to additional PAHs become available, they should be tested in the newer, micro-scaled multiplex immunoassay⁷⁹⁻⁸¹ and microarray ELISA⁸² formats.

6. *How have the scientific capabilities of collaborating scientists been improved?*

This project required sharing the unique expertise of all three labs. Each participant, from the PIs to the students and technical assistants, had to acquire basic knowledge of the rationales and methods used in the other labs. Dr. Roberts and co-workers gained new understanding and skills in molecular cloning, hapten design, and principles of immunoassays. Dr. Li's lab is setting up methods for scaled-up production of MAbs and recombinant antibodies, *in vitro* mutagenesis, and analysis of antibody binding kinetics. Dr. Karu and co-workers acquired expertise in hapten synthesis, antibody structure modeling, automated ligand docking, principles of marine and terrestrial ecotoxicology, and bioremediation by cooperative interaction of plants, lower eukaryotes, and bacterial communities.

7. *How has this research influenced our understanding in the area?*

There are basically two ways to engineer improved antibodies, as shown in **Figure 10**. The scheme on the left summarizes the approach we took with the Fab2LOX and Nissim phage display libraries. At right is the procedure we used beginning with rFabs 4D5 and 10C10.

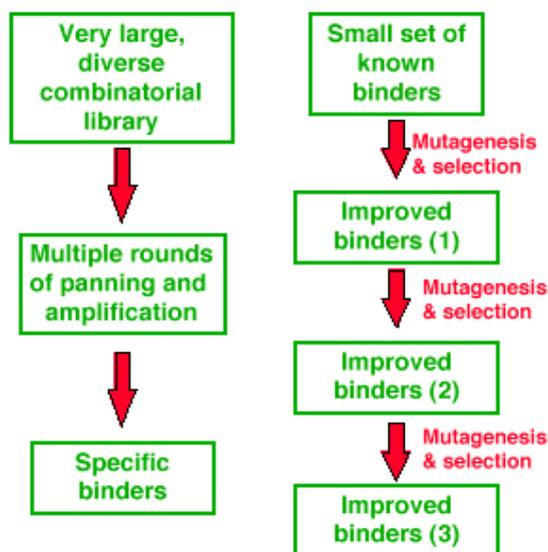


Figure 10. Flow chart for antibody engineering (after a seminar slide made by Prof. Andreas Plückthun, 1998).

When we began this project, very few semi-synthetic combinatorial antibody libraries were available, and even the researchers who developed these libraries had limited experience with target compounds outside their area of immediate interest. A widely held assumption was that functionally useful antibodies to virtually any target could be derived, if a library's repertoire was sufficiently large. Although antibodies to other small ligands were derived from the Fab2LOX and Nissim libraries, we could not recover antibodies to BaP or

fluoranthene from Fab2LOX, and scFvs selected from the Nissim library with naphthalene and phenanthrene haptens were weak binders.

Computational modeling and sequence analysis provided an explanation of why these human libraries were unlikely to yield antibodies to BaP and other PAHs. The unusually deep pocket for BaP binding in mouse rFabs 4D5 and 10C10 required substitution of the highly conserved tryptophan H47 with the less bulky side chains on valine and isoleucine. The mouse germline V_H genes include a sequence with this substitution, but the human V_H repertoire does not. rFab libraries that contain a significant percentage of members without both H and L chains are unlikely to yield useful binders, especially for hydrophobic ligands such as PAHs. Selection of the most functional antibodies from scFv libraries may be impaired if the linker between V_H and V_L is not optimal.

Antibody quality is also affected by the topology of binding sites, proper folding, and optimal fit at the V_H - V_L interface. Binding sites for epitopes on large proteins are generally broad, cuplike surfaces. Binding sites for peptides tend to be grooves, and sites for binding small molecules are deep pockets. We hope that our results are influencing display library developers and end users to pay much more attention to structural diversity, — i.e., shape, charge distribution, flexibility, orientation of the V_H and V_L domain interface, and properties of the linker in scFvs, — than to sheer size of the library they plan to use.

8. *What additional scientific or other hurdles must be overcome before the results of this project can be successfully applied to DOE Environmental Management problems?*

Scaled-up production and purification of recombinant antibodies remains at least as much an art as a science. Yeast and insect cell systems have proven to be much more efficient than *E. coli* for expressing certain antibodies. Technology for highly efficient expression and conditions that promote correct folding and disulfide bond formation are needed for all recombinant proteins. This problem is under intensive study worldwide. Improved expression systems are emerging rapidly. Many are commercially available and major innovations in this area can be expected. On the other hand, antibody scale-up is becoming less of a problem as assay systems are being miniaturized by microfluidics, microarrays, and similar technologies.

Antibodies and antibody fragments are the best understood and most widely used recognition molecules. However, the last several years have seen rapid development of other protein "scaffolds" for ligand-binding peptides. Antibodies and other recognition proteins would be much more useful if their resistance to physical and chemical denaturation and damage could be improved. Recent promising advances in this area include genetic engineering of the solvent-accessible surface and entrapment of the antibodies in sol-gel matrices.⁷⁵⁻⁷⁸

In the past, it was widely believed that the repertoire of naturally occurring and genetically engineered antibody structures imposed a ceiling on the affinity for particular ligands. As our project was nearing closure, this upper limit was exceeded more than 100-fold by application of a more random genetic sequence-shuffling technique known as molecular evolution.^{83,84} This technology will undoubtedly expand the potential applications of engineered antibodies.

9. *Have any other government agencies or private enterprises expressed interest in the project? Please provide contact information.*

This research program developed from a 1993-96 collaborative project between Dr. Karu and Professors David Walt and John Kauer at Tufts University in Medford, MA. That project, with Dr. Walt as the lead PI,

was funded by the Molecular Recognition program at the Office of Naval Research (ONR). The ONR Program Manager was Dr. Harold Bright.

The EPA has expressed interest in our research with regard to monitoring the progress of PAH and PCB phytoremediation at Superfund sites on Oahu. One such project is funded and in progress under Dr. Li's direction. Department of Defense (DOD) officials who expressed interest include Navy remediation managers at Pearl Harbor, and Army managers tasked with cleanup of the Makua Firing Range and other no-longer-used training areas on Oahu. Dr. Li also has collaborative studies under way with Professor Shannon Atkinson at the University of Alaska and Dr. Lee Ann Woodward in the Hawaii office of the U.S. Fish and Wildlife Service. These include analyses of PAH and PCB levels in turtles, albatrosses, and sea lions at the French Frigate Shoals, and in fish, coral, and sediments at Bikini atoll and other sites in the Marshall Islands.

PROJECT PRODUCTIVITY

To our knowledge, we produced the most comprehensive sets of PAH haptens presently available, using synthesis methods that are readily applicable to many similar compounds. The new knowledge from our project is applicable to production of improved antibodies and other types of recognition proteins for specialized analytical and sensing methods for many types of pollutants

Our results support the premise that the desired antibodies can be obtained by engineering, and from appropriately configured combinatorial libraries. Although we did not achieve our ultimate goal of producing superior PAH antibodies from large phage display libraries, we learned why. The most important work that was unfinished when the project ended involves creation and screening of a large number of random and domain-specific 4D5 and 10C10 phage display libraries. We created the starting material for an antibody library that may be able to distinguish PAHs in complex samples. Drs. Li and Karu are attempting to restart that work in Dr. Li's lab at the University of Hawaii.

PERSONNEL SUPPORTED

Institution	Name	Position
Univ. of Calif., Berkeley	Alexander E. Karu, Ph.D	Research Biochemist (Lead PI)
	Christopher W. Bell, Ph.D.	Postdoctoral researcher
	Tina Chin	Research specialist
	Bitao Zhao, M.S.	Research specialist
	Hui-I Kao, B.S, B.A.	Research Specialist
Univ.of Hawaii	Qing X. Li, Ph.D.	Associate Professor (Co-PI)
	Steven Thomas	Graduate student
	Kai Li, Ph.D.	Postdoctoral fellow
	Fengmao Guo, Ph.D.	Postdoctoral fellow
	Rongliang Chen, Ph.D.	Postdoctoral fellow
	Xiu-sheng Miao	Postdoctoral fellow
	Hu Li	Graduate student
	Mei Liu	Graduate student
The Scripps Research Institute	Victoria A. Roberts, Ph.D.	Associate Professor (Co-PI)
	Jean-Luc Pellequer, Ph.D.	Postdoctoral research chemist

Individuals who provided valuable input to the project, but received no financial support, included: Prof. Gary Rechnitz (Biosensors Center, Univ. of Hawaii); Prof. Paul L. Jokiel, (Univ. of Hawaii), Prof. Shannon Atkinson (Univ. of Alaska), Prof. Anne J. Feeney (The Scripps Research Institute), Prof. Robert Krieger (Univ. of Calif., Riverside), and Drs. Chris Swenson, Donald Palawski, and Lee Ann Woodward (U.S. Fish and Wildlife Service, Honolulu, HI).

PUBLICATIONS

This project resulted in sixteen peer-reviewed journal articles and symposium book chapters. These are listed here, and in the Literature Cited below.

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INTERACTIONS

In 1997, Dr. Robert Brizzolara at the Naval Surface Warfare Center, Carderock, MD, asked Dr. Karu if we could provide a fluorescent BaP hapten for tests of a sensor developed by Dr. Frances Ligler and co-workers at the Naval Research Laboratory in Washington, D.C. Bitao Zhao in Dr. Karu's group synthesized and verified the structure of a BaP-cy5 conjugate that we anticipated using in our project, and we sent a portion to Dr. Brizzolara.

Scientific meeting participation — oral and poster presentations:

1. Drs. Roberts, Li, and Karu participated in the First DOE EMSP Workshop, Chicago, IL, July 1998. Dr. Karu gave a short presentation, and we presented a poster. Karu, A. E.; Roberts, V. A.; Li, Q. X.: Engineered Antibodies for Monitoring Polynuclear Aromatic Hydrocarbons. Abstract in summary book, pp. 36-38, and poster: <http://home.osti.gov/em52/1998posters/id54546.pdf>
2. Drs. Roberts and Pellequer participated and presented a poster at the Second DOE EMSP Workshop, Atlanta, GA, April 25-27, 2000. poster: http://home.osti.gov/em52/NWS2000_Posters/id54546.pdf
3. Annual reports for this project may be found on the web:
1997 Year 1 progress report: <http://home.osti.gov/em52/emsp/54546.pdf>
1998 Year 2 progress report: <http://home.osti.gov/em52/1998projsum/54546.pdf>
1999 Year 3 progress report: <http://home.osti.gov/em52/1999projsum/54546.pdf>
4. Drs. Pellequer and Li both gave invited presentations at the Symposium on First Accomplishments of the Environmental Management Science Program (American Chemical Society 218th National Meeting, New Orleans, LA, Aug 22-26, 1999 (ACS Symposium #778). These papers were published as chapters in the ACS Book entitled, "Nuclear Site Remediation," cited above.
5. Dr. Roberts gave an invited presentation at the 221st American Chemical Society National meeting, April 2001; Roberts, V. A.; Pellequer, J.-L.; Karu, A. E.; Zhao, B.; Kao, H.-I.; Li, Q. X.; Li, K.; Thomas, S. PAH-specific recombinant antibodies as a test system for antibody engineering. Invited presentation by VAR: Session on Bioanalytical Techniques for Environmental Monitoring, Immunochemistry Summit IX, 221st American Chemical Society National Meeting, San Diego, CA, April 1-5; 2001; Agrochemicals Division, Abstract AGRO 111. http://schedule.acs.org/cgi-bin/ACS/pS.exe?FNC=Abstracts_AeventSearchResults_html_AGRO_111_172577_31725_PA

TRANSITIONS

Dr. Karu retired from the University of California at Berkeley, and is now a Visiting Scientist in the Dept. of Molecular Biosciences and Bioengineering at the University of Hawaii. He and Dr. Li are extending the work from this project to engineer functional variants of PAH-specific rFabs 4D5 and 10C10, and functional variants from S2B1, a rFab that is specific for the most toxic coplanar polychlorinated biphenyls (PCBs). Drs. Roberts and Pellequer are collaborating on a model of the PCB binding site in S2B1.

PATENTS

A licensing disclosure entitled "Haptens and Recombinant Fab Antibodies for Immunoassay and Immunoaffinity Recovery of Polynuclear Aromatic Hydrocarbons" will be filed with the Technology Licensing Offices at the University of Hawaii and University of California, Berkeley. The co-inventors will be Qing X. Li, Bitao Zhao, and Alexander E. Karu. An information copy will be forwarded to DOE as soon as the disclosure is completed.

FUTURE WORK

As time and resources allow, we want to fulfill the objective of preparing an antibody array for PAHs. We intend to develop a library of 4D5 and 10C10 mutants, if at all possible, and attempt to incorporate them in an array to monitor the progress of PAH bioremediation at a demonstration site on Oahu. With new funding, we intend to pursue development and application of engineered variants of the PCB rFab.

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