YEAR 1 PROGRESS REPORT

AWARD NO DE-FG07-96ER62316

PROJECT TITLE: **Engineered Antibodies for Monitoring of Polynuclear Aromatic Hydrocarbons**

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CO-PRINCIPAL INVESTIGATORS: Victoria A. Roberts (The Scripps Research Institute, La Jolla, CA)

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REPORTING PERIOD: 1 October 1996 - 30 Sept. 1997

AWARD PERIOD: 1 October 1996 - 30 Sept. 1999

STATUS OF BUDGET: The budget status for the 12 months beginning 1 October, 1886 is summarized in the table below.

	Year 1 appropriation	Expenditure	Indirect costs	Total Year 1 Expenditure	Balance remaining on Sept. 30, '97	Percent not spent by Sept. 30, '97
Berkeley	$135,766	$55,101	$47,913	$103,014	$32,752	24.1 %
Scripps	92,822	36,914		36,914	55,908	60.2 %
Hawaii	86,763	30,000*		30,000*	56,763	65.4 %
Total	315,351	122,015		169,928	145,423	

*Only $23,897 actually billed to UCB through 8/3l/97

Research and expenditures were delayed during the first four months of this project. Although the award became effective on Sept. 15, 1996, the University of California, Berkeley could not formally activate it and begin to process the subcontracts to Scripps and the University of Hawaii until early December 1996. This delayed recruitment, purchasing, and equipment procurement at all three institutions. Despite this, Drs. Li and Roberts accomplished most of their major Year 1 objectives described in the proposal. Dr. Karu's laboratory moved to the main Berkeley campus in December 1996 and January 1997, and Ms. Zhao, the primary technical person on the project was on maternity leave until April 1997. Dr. Li's laboratory was also relocating and was unable to begin work on the project until early March. Computer and software problems slowed some of Dr. Roberts's work early in 1997. To compensate for these delays Dr Karu's laboratory assumed some of the Li lab's hapten and conjugate synthesis tasks from April through July, and the two labs completed virtually all of the proposed hapten synthesis for the entire project. This enabled the Karu lab to proceed with combinatorial library screening, and the Li lab was able to begin developing PAH immunoassays and residue recovery methods in Year 1, rather than wait until Year 2. Dr. Li is employing a graduate student and another postdoctoral researcher to accelerate his part of the project. The Karu lab's antibody engineering tasks can be done more efficiently in Year 2. We propose to use the unspent Year 1 funds to employ a second person to perform the unfinished tasks beginning in January 1998.

TECHNICAL SUMMARY — YEAR 1

OBJECTIVE: The objective of this multidisciplinary project is to use molecular biological techniques to derive a set of antibodies with useful affinities and selectivities for recovery and detection of polynuclear aromatic hydrocarbons (PAHs) in environmental and biological samples. The long-term goal is to develop immunodetection methods that will be useful in biomarker research and regulatory monitoring of PAHs.

APPROACH The aims and approaches remain the same as in the original proposal. My laboratory cloned and characterized two PAL-I-specific recombinant Fab antibodies (rFabs). We are deriving new affinities and specificities for PAHs by mutagenesis of these rFabs, and by selection of new rFabs from combinatorial phage display libraries. Dr. Qing Li's group designed and synthesized PAH haptens that were essential for my laboratory's work. Dr. Victoria Roberts's group developed molecular models that suggested the mechanism of PAH binding and predicted mutations to alter it. Dr. Li's laboratory is using the recombinant antibodies we produce to develop immunoaffinity and immunoassay methods to quantify PAHs in environmental samples.

ACCOMPLISHMENTS: (Karu Laboratorv): The major progress in Dr. Karu's laboratory is summarized below. Separate reports by Drs. Roberts and Li are appended.

- PAH binding kinetics were measured by three different methods. The monoclonal antibodies (MAbs) from which we derived rFab antibodies had affinity constants and fast on- and off rates for benzolalpyrene and phenanthrene binding that are suitable for sensor applications. The rFabs had one-half to one-fourth the affinity of the parent MAbs. When compared with the MAbs, the rFab sequences had four different amino acids (including one extra glutamic acid) at the N terminus of the heavy chain variable domain, and three different amino acids at the N terminus of the light chain variable domain. These substitutions were introduced by the degenerate PCR primers used for gene amplification. Molecular modeling done by Dr. Roberts indicated that these substitutions may deform the PAH binding pocket. We are using ***in vitro*** mutagenesis to restore the original sequence. This should give the rFabs the same PAH binding affinity as the MAbs.

- In collaboration with Dr. Li's group, we designed and synthesized a set of PAH haptens representing the 3, 4, and 5-ring PAHs that are most water-soluble, have the highest vapor pressures, and are good indicators of the origin and composition of pollutant petrochemicals. Bitao Zhao in my laboratory prepared milligram amounts of ethylenediamine- and β-alanine- haptens of naphthalene, anthracene, phenanthrene, pyrene, fluorene, a similar hapten of 2-fluoranthene, and benzo[a]pyrene haptens with spacers at the 1 or 6 positions. The structure and purity of these was confirmed by mass spectrometry and NMR. Dr. Li's group synthesized 20 additional haptens. We prepared bovine serum albumin (BSA) and cytochrome c (CYT) conjugates of nine of these haptens, and coupled the conjugates to 5 μm-diameter paramagnetic glass beads for selection of antibodies displayed on phage.

- We used a mixture of the haptenated magnetic beads to select a combinatorial library enriched for PAH-specific rFabs from 1.2 x 10^{13} phage (displaying more than 10^{12} Fab sequences) in the semi-synthetic Fab 2LOX phage display library (Medical Research Council, Cambridge England). PAH binders in the first selection proved to be very rare —

on the order of 10^3 to 10^4 — in the original population of 1.2 x 10^{13} . Recovered phage were amplified by growth in *E. coli,* and depleted of antibodies that bound to the carrier proteins. Aliquots of this "PAH daughter library" were subjected to four cycles of amplification and panning with benzo[a]pyrene-6- and fluoranthene-3- haptens. This enriched the rFabs to these haptens by about 10^6-fold. These rFab sequences are being amplified *en masse* by PCR, inserted into an expression vector, and induced to express soluble rFab. Those that competitively bind benzo[a]pyrene and fluoranthene will be selected by enzyme immunoassay (EIA)

SIGNIFICANCE: Despite initial delays, several of the most important goals of the project were achieved during the past year. Twenty-nine PAH haptens were synthesized, representing all of the major PAHs that are likely to be detectable by immunoassay. All were prepared using only two general synthesis schemes - a Friedel-Crafts reaction and a Wittig reaction. These could be used to prepare additional haptens, including haptens of some heterocyclic PAHs, in the same way. Procedures for recovering PAH-specific antibodies from a very large combinatorial phage display library were worked out. Sequences that bind benzo[a]pyrene and fluoranthene were enriched sufficiently to make identification of the desired rFabs by standard EIA feasible. Dr. Li's group has formatted sensitive benzo[a]pyrene specific EIAs with one of our rFabs. They are now able to focus on making the EIA practical for use with real-world environmental samples.

The reduced affinity of the rFabs we cloned from two hybridoma lines, apparently a consequence of using degenerate PCR primers, is likely to arise in nearly all derivations of recombinant antibodies from cells. Our experiments will test whether this artifact may be corrected by *in vitro* mutagenesis. The molecular modeling done by Dr. Roberts's group indicates that PAH binding involves electrostatic complementarity, as well as shape, and may be a more general mechanism for antibody, enzyme, and receptor binding of other planar hydrophobic molecules. The models predict which amino acid side chains in the antibody participate in PAH binding, and how well different PAHs fit in the site. These predictions provide a rationale for our plans to engineer the antibodies, and a theoretical basis for understanding the results of Dr. Li's assay development with different competitor haptens.

WORK PLAN FOR YEAR II (Karu laboratory): The project will proceed essentially as described in the original proposal. Dr. Karu's laboratory will derive and characterize sets of rFabs that primarily bind to each of the nine PAH haptens. The engineering of rFabs 4D5 and l0C10, originally scheduled for Year 1, will be done in the coming year, based on the models and predictions made by Dr. Roberts's group. Several mutants of rFabs 4D5 and l0Cl0 will be constructed to test whether PAH binding is altered by changing the amino acids at the N-termini, or by changing the arginine and lysine on either side of the binding pocket. rFabs with useful properties will be scaled up, purified, and provided to Dr. Li's group for multi-analyte immunoassay development. Dr. Roberts's group will build structure models to reveal similarities or differences in PAH binding by various rFabs.

Personnel Involved: Dr. Karu, the PI, will continue to devote 15% of his time to the project. Ms. Bitao Zhao (MS. in Medicinal Chemistry, Univ. of Maryland) is an Assistant Specialist employed 100% time on the project since April 1997. She has been responsible for all of the hapten and conjugate synthesis and characterization, and all of the display phage selection.

Engineered Antibodies for Monitoring of Polynuclear Aromatic Hydrocarbons

Year-l Progress Report from
Department of Environmental Biochemistry, University of Hawaii
Report period: 10/l/1996 to 9/31/1997
Q.X. Li

1. Year-l Objectives:
   a. Synthesis, purification, and characterization of haptens.
   b. Conjugation of haptens to enzymes, carrier proteins and solid phases.
   c. Initial development of immunoaffinity and immunoassay methods.

2. Approach:

We have actively communicated with Drs. Karu (UC-Berkeley) and Roberts (The Scripps Research Institute) and their research groups. Hapten design was assisted by Dr. Roberts's computer modeling and interpretation of the binding site configuration of mAb 10Cl0.

3.Accomplishments:

a. Synthesis, purification, and characterization of haptens. We synthesized a library of 20 PAH haptens (Figure 1). These are derivatives of naphthalene, anthracene, phenanthrene, pyrene, fluoranthene, chrysene, fluorene and benzo[a]pyrene with spacers having a carboxylic acid terminus. The haptens were conjugated with various proteins and enzymes by standard methods. These conjugates are being used by Dr. Karu's laboratory to select recombinant Fab antibodies (rFabs) from a combinatorial phage display library, and by my laboratory to develop immunoassays for PAHs in environmental samples.

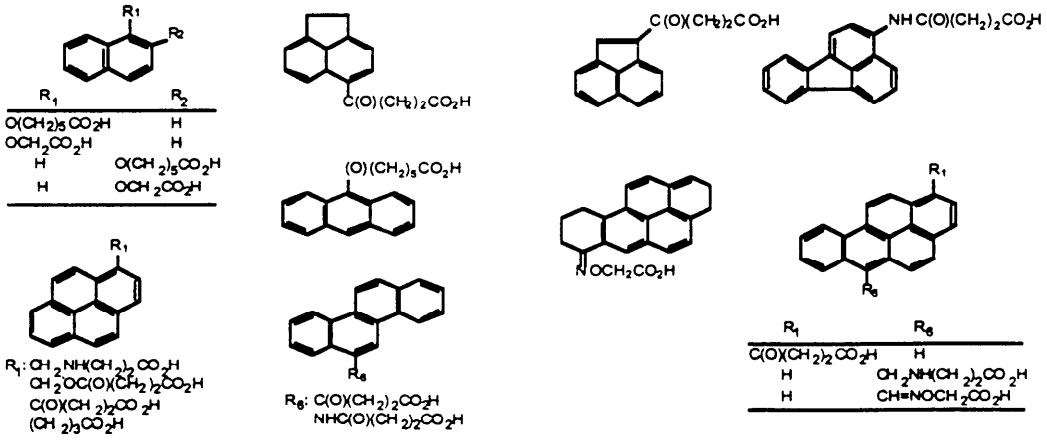

Figure 1. Structures of PAH haptens synthesized by Dr. Li's group

b. Initial development of immunoaffinity and immunoassay methods. Assays are being developed using PAH-specific monoclonal antibody (mAb) 10Cl0 and rFab 10C10. Figure 2 shows some of the conjugates that we are using to develop benzo[a]-pyrene immunoassays. We found that indirect competition EIAs could be performed using many of the 20 PAH-protein conjugates as competitors (Table below). We are now testing how these different coating antigens affect selectivity for PAHs.

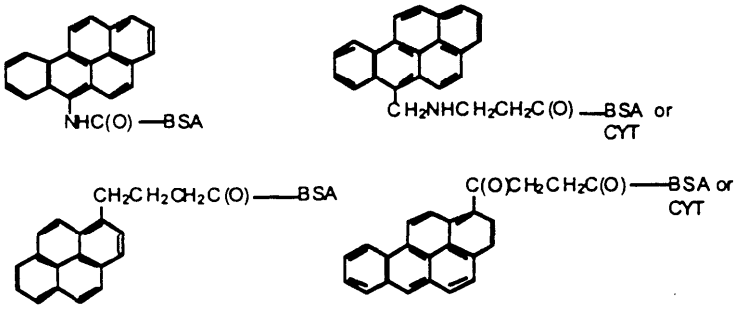

Figure 2. Conjugates used for benzo[a]pyrene immunoassay development

Figure 3 shows a typical inhibition curve obtained from indirect immunoassay. The concentration of benzo[a]pyrene that gives 50% inhibition ranged from 12 to 760 ppb, depending on the format and the coating antigen used.

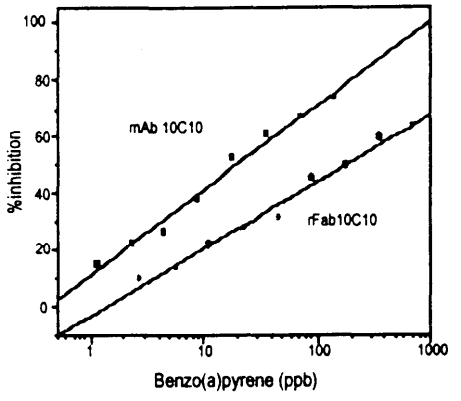

Figure 3. Inhibition by benzo[a]pyrene

Sensitivity of indirect competition EIA with MAb l0Cl0 using various PAH haptens as competitors.

PAH hapten-BSA (coating conjugate)	150 (ppb of benzo[a]pyrene)
pyrene	12
anthracene	16
fluoranthene	17
chrysene	21
phenanthrene	57
benzo[a]pyrene	83
fluorene	102
naphthalene	173

4. Significance: The library of PAH haptens and conjugates synthesized this year laid a solid base to accomplish all objectives proposed. The sensitive assays obtained with mAb 10Cl0 and rFab 10C10 indicate that recombinant antibodies will be very valuable reagents for PAH analysis.

5. Work Plan: We will continue our effort on development, validation and application of immunochemical methods for real world samples. Our year-2 objectives are:

a. Immunoaffinity methods for recovery and cleanup of PAH residues
b. Begin adapting solid phase extraction and supercritical fluid extraction for PAH sample preparation
c. Formatting and validation of multi-analyte PAH immunoassay
d. Synthesis and testing of candidate photoaffinity analogs of PAHs.

6. Personnel Involved: Dr. Q.X. Li (30% FTE, no salary support) is responsible for all work conducted at the University of Hawaii. This project supports Dr. K. Li (100% FTE, a post-doctoral fellow), Dr. R. Chen (l5%FTE, a junior chemist) and Mr. Steven Thomas 50% FTE, a graduate student). Ms. Mei Liu (50% FTE), a graduate student supported by a teaching assistantship from Department of Chemistry, University of Hawaii at Manoa also works on this project.

Principal Investigator: Victoria A. Roberts

Institution: The Scripps Research Institute, La Jolla CA 92037; Phone: (619) 784-8028; FAX: (619) 784-2289; E-mail: vickie@scripps.edu

Grant Title: Engineered Antibodies for Monitoring of Polynuclear Aromatic Hydrocarbons

Reporting Period: **1** Oct. 1996 - 30 Sept. 1997

Award Period: 1 Oct. 1966 - 30 Sept. 1999

Objective: The objective is to use molecular biological techniques to derive a set of antibodies for recovery and detection of polynuclear aromatic hydrocarbons (PAHs) in environmental and biological samples. In this component, the goal is to determine the structural basis of PAH binding to antibodies by constructing high-resolution three-dimensional models of the antibodies and to design structural modifications that will improve binding affinity and selectivity.

Approach: We are using molecular modeling techniques and protein structural analysis to construct high-resolution structures of antibody/antigen complexes and to understand the properties of PAH molecules. Analysis of these structures will direct the design of antibodies with enhanced antigen binding and selectivity, which will be engineered in Prof. Karu's laboratory, and selection of hapten linkers that do not perturb the chemical properties of the haptens to assist antigen design.

Accomplishments: To induce or isolate antibodies to small molecules, the small molecules must be attached to a carrier protein or a solid support through a linking functional group. Determining the effects of the linker on the properties of the small molecule itself, the target for detection, is essential for optimizing antibody binding. Therefore, we calculated shape and electrostatic properties for both benzo[a]pyrene (BP) and BP with attached linker (BP-linker), which was modeled by attaching a methyl urea group ($[CH_3-N(H)-C(=O)N(H)-]$) at the 6 position of BP. The attached linker significantly perturbs the electrostatic potential of the BP ring system (Figure 1). Three-dimensional, high-resolution structural models of the Fv (antigen-binding) domains of the two anti-PAH antibodies 4D5 and l0Cl0 were then constructed to determine the characteristics of their antigen-binding sites. The two sites are very similar in shape and electrostatic potential, displaying significant positive potential, as shown for the 4D5 antibody (Figure 2A). This positive potential better complements the more polarized charge distribution of BP-linker (Figure 2B). Thus, the linker probably greatly contributed to the induction of the Arg and Lys side chains that create the concentration of positive charge in the antibody binding pocket. The models also show that two aliphatic side chains in the bottom of the binding pocket (Val and Ile in 4D5 and Leu and Val in l0C10) are critical for the unusual deepness of the binding pocket.

Significance: Computer modeling of the structures of the 4D5 and l0Cl0 antibodies with bound antigen revealed sites for mutation to enhance PAH binding and selectivity. Mutation of Lys and Arg in the antigen-binding pocket to neutral side chains may significantly enhance binding of the neutral PAH molecules by decreasing the positive potential of the binding pocket. The orientation of BP in the 4D5 binding pocket indicates that a bound pyrene would be completely buried within the pocket, consistent with the almost identical binding characteristics of pyrene and BP to 4D5. Mutation of two side chains at the bottom of the pocket would substantially alter this fit, changing the selectivity of binding. Thus, the models have revealed specific sites for mutagenesis to enhance PAM binding affinities and alter selectivity, indicating routes for re-engineering these antibodies to be useful for detection of PAHs in environmental and biological samples.

Work Plan: Models for mutants with enhanced PAH binding will be built and analyzed to direct mutagenesis experiments in Prof. Karu's lab. PAH molecules with attached linkers will be analyzed to determine those linkers that least perturb PAH characteristics, which will be synthesized in Prof. Qing's lab. Models will be built of new anti-PAH antibodies obtained from phage display libraries.

Personnel Involved: Jean-Luc Pellequer: Postdoc; Construct and analyze antibody and hapten molecules.

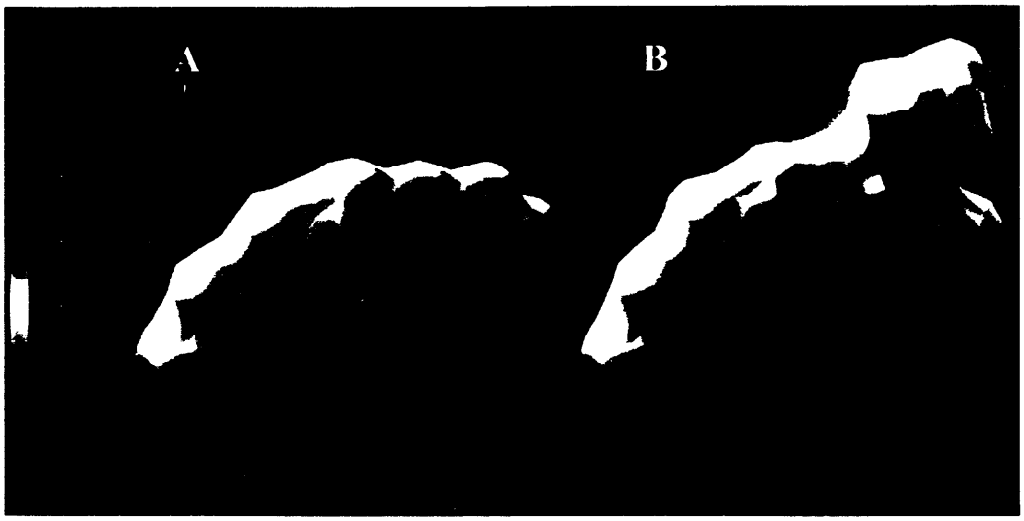

Figure 1. Comparison of electrostatic potential surfaces for BP and BP-linker. (A) BP displays weak negative electrostatic potential (red) above and below the planar ring system. (B) BP-linker shows a significantly different electrostatic potential surface. The linker (surfaced in upper right) that attaces BP to the carrier protein enhances the negative charge (red) on one side of the BP ring system and induces a small positive patch (blue) on the opposite side of the BP ring system. Geometry and partial atomic charges were calculated with the program Gaussian94. Surface electrostatic potentials were calculated with the program DelPhi. Energy scale is in kcal/mol.

Figure 2. Exquisite shape and electrostatic complementarity between the 4D5 antibody and bound BP-linker. The Cα trace and selected amino-acid side chains (colored tubes) are shown for the 4D5 antibody. (A) The surface of the 4D5 antigen-binding pocket is shaped so that it almost completely surrounds the ring system of BP-linker (center, green tubes with N (blue) and O (red) atoms of the linker shown as spheres). A strong positive electrostatic potential (blue) over much of the binding pocket surface is due to a Lys side chain (lower left) and an Arg side chain (right, center) on opposite sides of the pocket. A small region of negative potential (red) is present in the upper left of the binding pocket. (B) Same view showing the electrostatic surface of BP-linker. The region of negative potential caused by attachment of the linker may be responsible for induction of the Arg side chain on the right side of bound BP-linker. The small region of positive potential induced by the linker is adjacent to the region of negative potential found on the binding pocket in (A).