PROJECT ID NUMBER: 54546

PROJECT TITLE: Engineered Antibodies for Monitoring of Polynuclear Aromatic Hydrocarbons

DATE: March 6, 2000

PRINCIPAL INVESTIGATOR:

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NUMBER OF GRADUATE STUDENTS AND/OR POST-DOCTORATES ACTIVELY INVOLVED IN THE PROJECT:

Three graduate students and three post-doctorals

Research Objective:

Polynuclear aromatic hydrocarbons (PAHs) are a large class of structurally similar pollutants. Rapid, inexpensive, and high-throughput methods to identify and monitor PAHs are needed in several DOE focus areas, including human and ecosystem health effects, risk and exposure assessment, decontamination and decommissioning, and remediation. DOE has sponsored and participated in several demonstration projects in which commercial immunoassay kits proved useful and cost-effective for detection of PAHs and other pollutants. The emerging generation of sensors and residue recovery methods will require panels of antibodies with relatively subtle differences in cross-reactivity. This project is based on the premise that genetic engineering should be much more successful than conventional polyclonal and monoclonal antibody methods for developing these antibody panels.

One objective of this project has been to define the structural basis and mechanisms by which antibodies bind and cross-react with various PAHs. A second objective has been to use this information to produce recombinant antibodies with improved performance in analytical procedures that DOE can use. A third objective has been development of PAH residue recovery and cleanup methods that will be compatible with immunoassays, and make instrumental analysis faster, more accurate, and less expensive.

Research Progress:

This report summarizes work completed during the last 12 months. This includes seven months of the original three-year grant period, which ended on Sept. 15, 1999, and five months into a one-year no-cost extension.

Architecture of PAH binding sites. As described in previous reports, computational models were made of the PAH binding sites in two well-characterized murine recombinant Fab antibodies (rFabs), 4D5 and 10C10, that were originally evoked by a benzo[*a*]pyrene (BaP) hapten, and cross-reacted to different extents with 11 PAH haptens, and seven PAHs smaller than BaP. The sequences and models revealed five novel aspects of the binding sites: (1) The mouse germline genes that gave rise to 4D5 and 10C10 were identified as a set that is seldom expressed in B cells. This implies that their unusual structural motif was very stringently selected by the BaP hapten. (2) Although 4D5 and 10C10 differed by 23 amino acids, the binding site shapes and dimensions were virtually identical. Only three amino acid differences occurred in the binding pockets, and one of these, at position H93, did not contact bound PAH. The other sequence differences occurred in parts of complementarity-determining regions L1 and H2, that did not contact bound PAH, and on the antibody surface, where they could not directly affect PAH binding. (3) The binding pockets were unusually deep, because they had valine or leucine at framework position H47, instead of the bulkier tryptophan found in nearly all other mouse antibodies. (4) The binding pockets were formed partly by cationic side chains of lysine (Lys) L89 and arginine (Arg) H95. These created patches of positive charge, positioned where they could interact with the π electrons on either side of bound PAHs. (5) Computational ligand docking results were consistent with immunoassay data indicating that BaP and BaP haptens could be bound in two different orientations.

To test whether the Lys L89 and Arg H95 side chains had a role in PAH binding, in vitro mutagenesis was used to replace them with amino acids that have relatively neutral side chains of about the same length — methionine and glutamine, respectively. Performance of the original, single, and double mutant rFabs was compared by enzyme-linked immunosorbent assay (ELISA). The L89 mutant competitively bound soluble benzo[a]pyrene (BaP), pyrene, and fluoranthene, with half-maximal inhibition (I_{50}) values comparable to that of wild-type 4D5, but the mutant did not bind phenanthrene, anthracene, fluorene, or chrysene. The H95 single- and L89-H95 double mutants abolished binding to all PAH haptens. Thus, Lys L89 affected cross-reactivity, and Arg H95 was essential for PAH binding.

Three single-chain Fv antibodies (scFvs) that bound a naphthalene hapten, and three that bound a phenanthrene hapten, were selected from the very large Nissim phage display library. One scFv, Nap-2, competitively bound pyrene

as well as naphthalene. PAH binding sites in the scFvs were shallower than those in the mouse rFabs. However, positively charged side chains of arginines at positions L91 and H95 were located symmetrically on either side of the binding sites, in positions that should allow cation-• interactions with PAHs. This inference was supported by computational docking results which indicated that pyrene was bound by Nap-2 scFv in approximately the same orientation as it was in rFabs 4D5 and 10C10.

The unusually deep binding pocket for BaP and other PAHs in rFabs 4D5 and 10C10 originated from a rarely expressed set of mouse germline V_H genes that had Val or Leu, instead of Trp, at position H47. Virtually all known human germline V_H genes have a Trp, and none have Val or Leu, at H47. Consequently, the human germline V gene repertoire may not include sequences that can form such a pocket.

Method development. Supercritical CO_2 extracts of materials such as marine coral contain different types and amounts of substances that complicate or prevent direct instrumental analysis of PAHs. GC-MS analysis (total ion monitoring) of PAHs of the crude SFE extracts of corals produced very complex chromatograms with poor separation of the target analytes from interferents. Dr. Li's laboratory developed a protocol for immunoaffinity cleanup (IAC) of supercritical CO_2 (SFE) extracts using immobilized MAb 4D5. Samples prepared in this way were compatible with both GC-MS and ELISA analysis. A pilot study was done to compare the amounts and types of PAHs recovered from marine coral by SFE-IAC, and by pressurized fluid extraction (PFE) using dichloromethane : acetone :: 1:1 (v:v) at 2,000 psi and 100 •C. PFE extracts of coral samples fortified with 12 different 2-5 ring PAHs gave average recoveries of 65% for naphthalene and 87-112% for the other PAHs. Similar results were obtained by SFE-IAC.

1-Hydroxypyrene, also known as 1-pyrenol, is an established biomarker of PAH exposure secreted as glucuronides and other conjugates in urine. Dr. Li's group also developed an improved protocol for SFE of 1-hydroxypyrene bioconjugates in urine, followed by high-pressure liquid chromatography (HPLC). Addition of triethylamine to the samples before HPLC blocked secondary retention effects, shortened analysis time to about 20 min per run, and lowered the limit of detection to about one-third of that obtained in the original method.

As part of a collaboration with biologists in the U.S. Fish and Wildlife Service, *Porites* coral samples were obtained from Tern and Trig islands, a relatively pristine, restricted access area northwest of Hawaii, and from Kaneohe Bay, a heavily used area on Oahu that has been subjected to petrochemical pollution for decades. Unspiked samples from Tern and Trig islands contained chrysene and benzo[a]anthracene at 0.63 and 0.52 ug/g dry weight, respectively, and smaller amounts of eight other PAHs. PAH concentrations in Kaneohe Bay corals were roughly five-fold higher.

Significance and Implications:

Although we have not yet made a panel of improved antibodies, this project has produced very specific information that should enable us and others to do so. The PAH binding sites in the antibodies we studied have a distinctive structure, and binding is at least partly due to a cation-π ınteraction. Similar bonding has been described in ligand interactions with several receptors and enzymes, and in binding of phosphocholine by antibody McPC603. However, all of these involved a cationic ligand and aromatic amino acid side chains in the binding protein. To our knowledge, PAH binding by the mouse rFabs 4D5 and 10C10 and (by inference) the human Nap and Phen scFvs, are the first examples in which an aromatic ligand is stabilized by positively charged side chains in the binding protein. These findings pose intriguing possibilities for how PAHs may be bound by the aryl hydrocarbon receptor, recently described BaP-binding proteins such as glycine N-methyltransferase, plasma lipoproteins, and PAH-metabolizing enzymes important for bioremediation.

One of our most important findings was that there were no human germline sequence counterparts to the mouse germline sequences in rFabs 4D5 and 10C10. This may explain why we were unable to recover new BaP-binding antibodies from the human Fab 2LOX rFab and Nissim scFv phage display libraries. Despite their very large sequence diversity. the human libraries lacked the necessary structural motif.

Our binding site models and docking results suggest several options for mutagenesis to change cross-reactivity, as well as affinity. A library made by mutagenesis of murine rFabs 4D5 or 10C10, should be a rich source of useful variants.

Sample preparation and cleanup methods are the most expensive, error-prone and time-consuming elements in any environmental analysis project. Dr. Li's method development is making immunoassay and immunoaffinity methods practical for an ever-increasing number of applications.

Planned Activities:

With less than six months remaining, two practical issues will receive priority. Drs. Roberts and Pellequer will attempt to obtain a definitive X-ray crystallographic structure of rFab 4D5, to confirm predictions of the binding site

models and obtain more accurate data to guide the mutagenesis strategies. Dr. Li's group will continue to improve the SFE-IAC and other protocols for PAH metabolite analyses in specific DOE applications. Scaled-up expression of the mouse rFabs and mutants is essential for both of these tasks, as well as for the first steps toward deployment for practical uses.