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Engineered Antibodies for Monitoring of Polynuclear Aromatic Hydrocarbons

Alexander E. Karu
University of California
235 Stanley Hall, MC 3206
Berkeley, California 94720-3206
Phone: 510-643-7746
E-mail: hyblab@socrates.berkeley.edu

Victoria A. Roberts
The Scripps Research Institute
10550 North Torrey Pines Road
La Jolla, California 92037
Phone: 619-784-8028
E-mail: vickie@scripps.edu

Qing Xiao Li
University of Hawaii
329 Henke Hall
1800 East-West Road
Honolulu, Hawaii 96822
Phone: 808-956-2011
E-mail: qingl@uhunix.uhcc.hawaii.edu

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Alexander E. Karu, University of California at Berkeley

Victoria A. Roberts, The Scripps Research Institute
Qing Xiao Li, University of Hawaii

Research Objective

The long-term goal of this project is to develop antibodies and antibody-based methods for detection and recovery of polynuclear aromatic hydrocarbons (PAHs) and PAH adducts that are potential biomarkers in environmental and biological samples. The inherent cross-reactivity will be exploited by pattern recognition methods. Dr. Karu's laboratory uses new haptens representing key PAHs to derive recombinant Fab (rFab) and single-chain Fv (scFv) antibodies from hybridoma lines and combinatorial phage display libraries. Computational models of the haptens and combining sites made by Dr. Roberts's group are used to guide antibody engineering by mutagenesis. Dr. Li's laboratory develops enzyme immunoassays (EIAs), sensors, and immunoaffinity methods that make use of the novel haptens and antibodies for practical analytical applications in support of DOE's mission.

Research Progress and Implications

This report summarizes work completed in one and one-half years of a 3-year project, with close collaboration between our three research groups. Dr. Alexander Karu's laboratory: We proceeded with the two strategies described in our original proposal. Site-directed mutagenesis was used to correct differences in the rFab N-terminal amino acids that were introduced by the degenerate PCR primers used for gene amplification. The binding constants of the rFabs with the corrected sequences will be compared with those of the parent MAbs, and should be very similar. The 4D5 and 10C10 heavy and light chain sequences are being moved to the pCOMB3H phagemid vector to facilitate selection of new engineered mutants.

We attempted to recover rFabs that bound the benzo[a]pyrene-6- and fluoranthene-3- haptens from the semi-synthetic Fab 2LOX phage display library (Medical Research Council, Cambridge, England). However, PCR analyses showed that only about 10% of the sequences in the original library were intact rFabs. The library had to be depleted of phage that bound to plastic or biotin, as well as phage that lacked a Fab L chain. The remaining display phage that bound the PAH haptens were enriched by about 10^6-fold, but all proved to be nonspecific-binding sequences. Consequently, work with this library was discontinued, and essentially the same protocol was applied to screen the Nissim single-chain Fv (scFv) library, which contained about 10^8 sequences. Six naphthalene-binding scFvs and six phenanthrene-binding scFvs were recovered. We are presently characterizing the PAH cross-reactivity and competitive binding by these antibodies, and panning the Nissim library with the other PAH haptens.

Dr. Qing Li's laboratory: The sensitivity and cross-reactivity of the 4D5 and 10C10 MAbs and rFabs from Dr. Karu's laboratory were measured in EIAs with the various PAH haptens developed earlier. Direct competition EIAs were performed with hapten-horseradish peroxidase conjugates of pyrene and benzo[a]pyrene (BP). Indirect EIAs using different coating antigens were most sensitive: pyrene-1-BSA for the MAbs, BP-6-BSA for rFab 10C10, and phenanthrene-10-BSA for rFab 4D5. EIAs with the rFabs were much more sensitive for 4- and 5-ring PAHs (I50 = 170-230 ppb) than for 2- and 3-ring PAHs (I50 > 3700 ppb). The results showed that selectivity as well as sensitivity of these antibodies to PAHs can be altered over a substantial range by changing the assay format and competing hapten.

Significant progress was also made toward reliable PAH immunoanalysis of environmental and biological samples. Indirect competition EIA was used to compare recovery of BP spikes from 0.5-151 ppb (2-600 nM) in phosphate buffer, tap water, unfiltered Ala Wai canal water, and urine. Recoveries averaged 88 ± 6% to 95 ±13% in buffer and water, and 111 ± 17% in urine. No significant matrix effects were observed with these samples, other than the slightly higher bias in the urine. The EIA results with these samples correlated closely (slope =0.97; r2= 0.99) with values obtained by gas chromatography (GC).

We developed a versatile chelating agent-assisted supercritical fluid extraction method that allowed quantitative (87-106%) recovery of the nonpolar PAHs as well as polar chlorophenoxy pesticides aged in clay soils. This method is being applied to determine bioavailability of PAHs in contaminated harbor sediment.

We also began a study of PAH bioaccumulation in marine coral. Methods were developed for Soxhlet extraction of organics from 50-gram samples into hexane. The recovery of PAHs added as pre-extraction spikes was 85-108%, determined by GC/FID and GC/MS. Porites compressa (a non-endangered species) from unpolluted waters contained 19-23 ng of naphthalene and 114-153 ng of phenanthrene per gram. Immunoaffinity columns prepared by coupling MAb 4D5 IgG to CNBr-agarose gave 68-85% recovery of BP standards in phosphate buffer. We are adapting the immunoaffinity procedure for rapid concentration and cleanup of coral extracts for instrumental PAH analysis.

In collaboration with Dr. Garry Rechnitz, we developed a capacitive immunosensor using MAb 10C10 for detection of PAH-protein adducts as potential biomarkers. The MAb was immobilized on a gold electrode through a self-assembled monolayer of cystamine. Binding of 0.01-6.0 μM BP-BSA and pyrene-BSA conjugates was detected by linear sweep voltammetry as a decrease in the charging current. This and other sensor formats are being tested for measurement of PAHs in solution.

Dr. Victoria Roberts' laboratory: We constructed and analyzed three-dimensional models of the PAH-binding rFabs 4D5 and 10C10, calculated PAH and PAH-linker molecular properties, and performed intermolecular docking studies to understand PAH-antibody interactions. From these results we identified targets for site-directed and combinatorial mutagenesis of the antibodies.

Dr. Li's laboratory demonstrated that the 4D5 and 10C10 antibodies were able to bind BP haptens with either a succinic acid linker at carbon 1 (BP-1) or an isocyanate linker at carbon 6 (BP-6). To better understand these interactions, the shape and electrostatic properties of both haptens were calculated by quantum mechanical methods, and docking to the energy-minimized antibody was simulated using the program AUTODOCK. Linker attachment at C1 caused little perturbation of the electrostatic potential of the BP ring system, unlike the linker on BP-6. Thus, the succinic acid linker used in BP-1 hapten should be better than the isocyanate linker of BP-6 for selecting antibodies that are optimized to bind BP.

Fifty to 100 docking experiments were done for each molecule. Closely docked positions were clustered together. The most energetically favored cluster found for BP-6 hapten closely matched (within 1 Å) the docked position of the BP-6-isocyanate hapten that evoked the antibody. BP-1 had only one favorable docked position. BP itself docked to rFab 4D5 in four possible binding modes with roughly equal energy, of which two were similar to BP-6, and two were similar to BP-1. Therefore, the 4D5 binding site structure is consistent with hapten BP-1 binding almost as strongly as BP-6. The multiple binding modes found for BP are consistent with the binding constants for different PAHs determined by Dr. Karu's group, and the EIA results obtained by Dr. Li's group with different PAH haptens. The docking results suggested that the binding pocket's shape may be further optimized for better selectivity among the PAHs. The hydrophobic side chains at the bottom of the pocket appeared to be particularly critical for selectivity. Combinatorial mutagenesis of the two segments of the variable heavy chain containing these side chains should produce variants with different PAH binding profiles. The docking studies also revealed possible binding sites for BP that are near, but not in, the binding pocket, providing an explanation for the relatively strong binding of PAH hapten conjugates and PAH metabolite-protein adducts. One molecule of a conjugated protein may present more than one hapten or adduct residue to a single antibody molecule, resulting in binding external to, as well as within the specific primary binding site. These loci may also account

for the large percentage of weak non-specific hydrophobic binders that Dr. Karu's group recovered when they used PAH-hapten conjugates to pan combinatorial phage display Fab and scFv libraries.

Planned Activities

During the next six months, the new naphthalene and phenanthrene-binding scFvs will be purified and provided to Dr. Li's laboratory, where they will be rigorously characterized and used in multi-analyte EIAs, sensors, and immunoaffinity methods applicable to real-world samples. The scFv DNA sequences will be used by Dr. Roberts to develop and interpret models of these new PAH binding sites. Dr. Karu's group will continue to derive additional scFvs to other PAH haptens from the combinatorial library, and to select new variants by combinatorial mutation of the 4D5 and 10C10 Fab antibodies.