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Author	Vanderlaan, Martin
Corporate Author	Lawrence Livermore National Laboratory, Biomedical Sc
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QUARTERLY TECHNICAL REPORT
(January-March, 1986)

IMMUNOASSAYS FOR DIOXIN

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Prepared by:

Martin Vanderlaan

Biomedical Sciences Division
Lawrence Livermore National Laboratory
P.O. Box 5507
Livermore, CA 94550

For:

William Budde
Environmental Protection Agency
26 W. St. Claire St.
Cincinnati, OH

Summary of Progress

Three major accomplishments have been made during the past three months:

1. The binding specificities of three monoclonal antibodies, DD-1, DD-3, and DD-4 to dioxins have been determined for sixteen compounds. All three antibodies have a strong preference for the tetrachloro isomers of dioxin and dibenzofuran relative to more highly chlorinated dioxins and dibenzofurans, and polychlorinated biphenyls. Large lots of these monoclonal antibodies have been produced.

2. Two additional monoclonal antibodies, DD-5 and DD-6, have been selected. The binding specificities of these antibodies have not yet been characterized extensively, although they recognize 2,3,7,8-tetrachloro-dibenzodioxin. Large scale antibody production and characterization of these monoclonal antibodies is in progress.

3. An improved immunoassay protocol has been developed using DD-1. Unlike the original method, where the dioxin was suspended in water using detergent, we now solubilize the dioxin by adsorbing it onto protein. This new method is more reproducible and takes less time to complete than the old method, and retains a detection limit of about 1ppb.

All of these accomplishments are well ahead of our projected milestone timetable.

Detailed Report of Progress

Three hybridomas, DD-1, DD-3, and DD-4, have been produced. Briefly, the protocol used to generate these hybridomas was to immunize mice with 1-amino-3,7,8-trichloro-dibenzodioxin (A-triCDD) conjugated to bovine serum albumin (BSA). A total of 9 injections of 10 µg/injection in adjuvant were given over 10 months. A tenth injection was given four days before taking the spleen cells for fusing. DD-1 and DD-3 came from the same BALB/c mouse spleen. DD-4 came from a Biozzi mouse. In both cases, the splenocytes were fused with the SP2/0 mouse myeloma cell line, and hybridomas were initially selected for their abilities to distinguish A-triCDD-rabbit-serum-albumin (A-triCDD-RSA) from rabbit serum albumin (RSA). Next, the antibodies produced by these cells were tested for their ability to recognize 2,3,7,8-TCDD in solution at concentrations of 100 ppb or less. The clones DD-1, DD-3, and DD-4 were selected for further culture and characterization because they had the greatest sensitivity to free TCDD. The cells were subcloned twice to insure that they were "monoclonal", and stock cultures have been frozen for long-term storage. About 100 mg of each monoclonal antibody has been produced and purified.

The binding specificities of the monoclonal antibodies secreted by these clones have been determined using a competition Enzyme Linked Immunosorbent Assay (ELISA).

The results are shown in Figure 1 and Table I, while the details of the ELISA method are discussed at the end of this section. Figure 1 shows representative competition data for DD-1 using several chlorinated compounds as competitors. From each of these curves, and others like them for DD-1 and DD-4, one may read the concentration required to inhibit antibody binding by 50% (I_{50}). Table I lists the I_{50} for all compounds tested so far with the DD-1, DD-3, and DD-4 antibodies. In reporting these values, factors of two are about the limit of significance. All three antibodies have highest affinity for tetrachlorodibenzodioxins, with highest affinity for the mixed isomers (1,2,3,7/1,2,3,8). They have slightly less affinity for the 2,3,7,8-TCDD and 2,3,7,8-TCDBF. DD-4 has some reactivity with the 1,2,3,6,7,8-hexa-CDD. Up to the highest concentration tested (100 ppb) all three antibodies either do not react or react only marginally with hexachloro-DBF, octachloro-DBF, octachloro-DD, and PCB's. The binding specificities of these three antibodies are highly desirable, since they prefer the most toxic of the dioxin and dibenzofuran isomers.

It is our experience that different mice will produce clones with different binding specificity and affinity. Each mouse appears nearly unique in the way it responds to an immunogen. Multiple clones from the same mouse are often quite similar, as is exemplified by DD-1 and DD-3. As such, we are interested in deriving a set of monoclonal antibodies from different mice, and DD-4 is the first example of that. We have selected two other hybridomas, DD-5 and DD-6 from different mice using the same basic protocol for the first three clones. It is premature to comment on their specificities, except to say that all recognize 2,3,7,8-TCDD. A detailed characterization of their binding properties is in progress.

A large part of the success that we have had in characterizing the binding specificities of our monoclonal antibodies comes from having developed a reliable competition ELISA protocol. Our current assay starts from stock solutions at 10 ppm in hexane of the various compounds listed in table I. 10µl of hexane solution is aliquoted into a small vial, and 500µl of phosphate buffered saline with 0.1% BSA is added. The vial is capped and placed in an ultrasonic cleaning waterbath for two hours. Apparently the dioxin, because of its hydrophobicity, becomes adsorbed to the BSA. Two hours of sonication gives a more reproducible assay than does one hour. During the sonication the hexane appears to evaporate completely, and one is left with an aqueous solution containing the dioxin. While the dioxin is mixing with the BSA solution, a microtiter plate is coated with A-triCDD-RSA. A two-fold dilution series of sonicated dioxin-BSA is then made, covering the range 100 ppb-0.1 ppb. In each well of the plate 100 µl of the sonicated dioxin-BSA solution is mixed with an equal volume of antibody. The antibody partitions between the dioxin adsorbed to the BSA, and the A-tri-CDD-RSA on the plate.

This assay has several advantages over the detergent (Cutscum) micelle solubilization method developed by Dr. Albro and adapted to ELISA by us initially. The detergent

method gave irreproducible results for us, suggesting that the formation of dioxin-micelles was variable or that the interaction of the dioxin-micelles with antibody was variable. Using BSA as a carrier seems to have alleviated this problem, and our competition curves are nearly identical run-to-run. In addition, the initial protocol called for drying the dioxin and Cutscum under nitrogen which was time consuming. The direct mixing of dioxin solution with BSA solution eliminates this step, although it does seem that an extended period of sonication is needed to achieve consistent results.

Plans for the Immediate Future

The binding specificity of DD-1, DD-3, and DD-4 will be characterized further in competition ELISA. In addition we will completely characterize the binding of our other candidate monoclonal antibodies, DD-5 and DD-6. We will add to the list of test compounds tested unchlorinated and lightly chorinated (mono, di, and tri) dibenzodioxins and dibenzofurans. It is important for us to determine quantitatively how well these antibodies can discriminate TCDD from other chemicals that could be present at much higher concentrations in samples. For example, in transformer dump sites the concentration of PCB's may be several thousand-fold more concentrated than the TCDD. The degree of specificity that we have observed so far is encouraging, but we have not evaluated the potential for interference in the immunoassay of very high concentrations of PCB's. Similarly, in herbicide and chemical waste sites 2,4,5-T, hexochlorobenzine, or DDT may be vast excess. As such, we will test other chlorinated hydrocarbons, such as DDT, 2,4-D, 2,4,5-T, hexachlorobenzine, and trichlorophenol in concentrations up to 10 ppm.

The immunoassay protocol will be optimized using ^{14}C-dioxin. The radiolabel will be followed during the ELISA assay to assure ourselves that we are quantitatively removing the dioxin from spiked soil samples, concentrating it in hexane, and adsorbing it onto the BSA.

Publications

Three meeting abstracts have been submitted based on this work, and copies are attached. Two are to the IUPAC meeting on Pesticide Chemistry in Ottawa, Canada, August 1986. Bruce Watkins and Martin Vanderlaan will attend that meeting. The third is to a workshop on biotechnology being sponsored by the Army in Cashiers, North Carolina,and Larry Stanker will attend.

1. Vanderlaan, M., B. Watkins, R. Devivar, and L. Stanker. Enzyme Immunoassay for Dioxins using Monoclonal Antibodies. Poster presentation IUPAC meeting on Pesticide Chemistry.

2. Vanderlaan, M., and J. Van Emon. Monoclonal Antibodies and Immunoassays for

Chemical Residue Analysis. Invited plenary session presentation at the IUPAC meeting on Pesticide Chemistry.

3. Stanker, L.H., M. Vanderlaan, B. Watkins, and J. Van Emon. Immunoassays of Trace Organics. Workshop presentation at an Army Biotechnology Workshop, April, 1986.

Figure 1

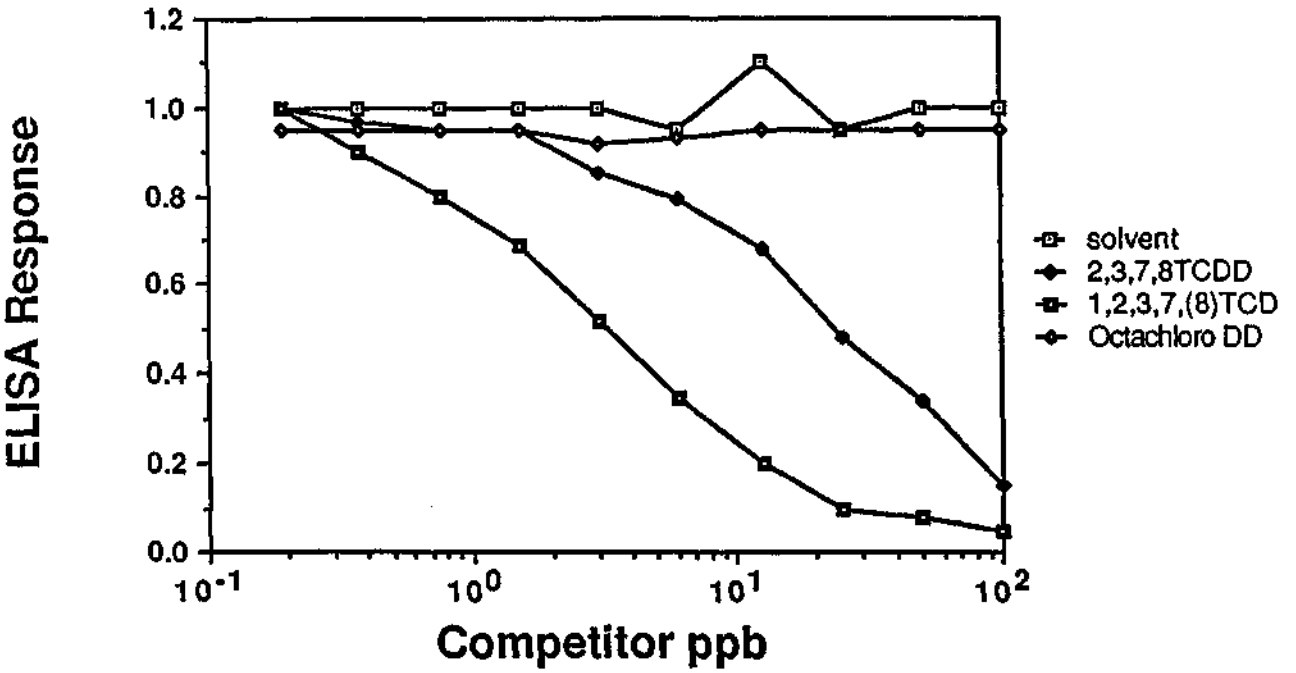

Figure 1 shows the results of a competition ELISA using monoclonal antibody DD-1. A-TriCDD-RSA was adsorbed onto the surface of the wells of a microtiter plate. The various competitors listed were suspendend in saline containing bovine serum albumin (BSA). These solutions were made by sonicating 10 µl of dioxin in hexane with 500 µl of saline-BSA. The dioxins appear to be adsorbed to the BSA and thereby remain suspended in the aqueous solution. A dilution series of these dioxin-BSA solutions was then made covering the range of 100-0.2 ppb, and placed in the microtiter plate wells. An equal volume of DD-1 monoclonal antibody was then added, and allowed to react for 1 hour. The antibody binds either to the A-TriCDD-RSA on the plate or the dioxin-BSA in solution. After the hour, the solution phase is removed, the plate washed, and it is reincubated for an hour with peroxidase-conjugated goat-anti-mouse-immunoglobulins. A second washing is then done, and substrate (ABTS) added for the peroxidase. The enzyme-antibody conjugate and substrate function as a "developer", allowing the visualization of the DD-1 bound to the A-triCDD-RSA on the plate. Results are then expressed as a fraction of the response in wells with no competitor. In this example, DD-1 does not react with octachlorodibenzodioxin. At about 20 ppb of 2,3,7,8-TCDD the relative ELISA response is half of control, meaning that half the DD-1 antibody bound to the solution phase dioxin-BSA and half bound to the A-triCDD-RSA on the plate. For 1,2,3,7(8)-TCDD, 50% inhibition occurs at about 4 ppb, indicating that DD-1 prefers 1,2,3,7(8)-TCDD to 2,3,7,8-TCDD.

	I_{50} (ppb)		
Competitor	DD-1	DD-3	DD-4
2,3,7,8-TCDD	20	5	25
1,2,3,7(8)-TCDD	4	1	3
1,2,4 Tri-CDD	35	>100	>100
1,2,3,6,7,8-hexa-CDD	>100	>100	50
Octachloro -DD	>100	>100	>100
2,3,7,8-TCDBF	10	5	25
1,2,3,4,8,9 hexa-CDBF	100	>100	>100
Octachloro-DBF	>100	>100	>100
2,2',4,6-TCBP	100	>100	>100
3,3',4,4'-TCBP	100	>100	>100
2,2',3,4,5-penta-CBP	>100	>100	>100
2,2',3,4,4',5-hexa-CBP	100	>100	>100
2,2',3,4,5,5',6-CBP	>100	>100	>100
2,2',3,3',4,4',6-CBP	>100	>100	>100
2,2',3,3',4,5,6,6'-CBP	>100	>100	>100
2,2'',3,3',4,4',5,5'-CBP	100	>100	>100