PROJECT 2: Covariates, Confounders and Consistency: Characterizing the Vietnam Veteran for Epidemiological Studies

BACKGROUND AND OBJECTIVES

While all analytic epidemiological studies have some conceptual elements in common, every study population offers unique challenges to the research scientist. Vietnam veterans are no different in this respect. Before undertaking any study, the investigator needs to understand key features of the target population that may influence choice of study design, techniques, and analytic methods. Such data for Vietnam veteran populations have been especially elusive, and many studies have failed to meet objectives because of lack of appreciation of these issues. (Zahm, 1995) One example is the naïve use of inappropriate surrogate measures of herbicide exposure. Another is inattention to or ignorance of the nature of exposure to combat, which is a defining variable for many serious chronic health outcomes, including PTSD, as was convincingly demonstrated by the National Veterans Readjustment Study (e.g. Jordan et al.; 1991).

In 1984 we carried out a cross-sectional survey of 12,500 members of The American Legion, using a comprehensive questionnaire that included items/measures on military herbicide exposure; combat experience and other key variables. Our findings on combat and PTSD were strikingly similar to that of Jordan and co-workers. This project entails resurveying the 1984 Legion cohort in order to reach the following objectives:

1.To create a pool of well-characterized veterans from which meaningful samples of subjects can be drawn for use in Project 3 "*Evaluation and Standardization of Military Personnel Records for Epidemiological Studies*" and Project 4 "*Biomarkers of TCDD Exposure in Vietnam Veterans,*" as well as to establish a sizable cohort for future epidemiological studies

2. To gain useful insights into the major variables, including herbicide handling, combat exposure, and PTSD, that can be expected to materially affect the conceptual framework, design, conduct, and analysis of any epidemiological study of Vietnam veterans

3. To investigate the relationship of these key variables to integral design issues of non-response which may limit an investigator's ability to find and locate Vietnam veterans and controls in any future studies

4. To address issues of generalizability by investigating the importance of variables, whose significance for Legionnaire studies has previously been established, to study groups of Vietnam veterans who either were under-represented (Blacks and Hispanics) or not included (women) in our initial study, and who may also been poorly represented in other studies

5. To examine issues of veteran recall. Because of the great length of time that has elapsed since the Vietnam Era and the difficulties in assigning precise exposure measures, the issue of respondent recall is particularly important for any future epidemiological studies of Vietnam Veterans. Understanding respondent recall is , however, complicated by the problem that veterans' reports of military service may always have been inaccurate, regardless of when the questioning took place. We deal with

this issue in Project 3, which examines concordance between reports and military records. Here we are specifically concerned with comparing longitudinal responses, which is one major reason that revisiting the cohort is a valuable undertaking. If recall is poor, especially if the poor recall is focused on particular items or measures, the validity of any epidemiological study that relies on these measures would be compromised. Thus, for all Veterans with in-country experience in Vietnam who responded to Time 1 (1984) and Time 2 (1998) surveys, we will compare responses to questions on focus variables.

In order to accomplish these objectives we will consider several methodological issues that, in our experience, strongly affect epidemiological investigations of Vietnam veterans, and that future researchers will need to appreciate when designing sound studies. By utilizing the original population of 12,500 members of The American Legion plus some additional Veterans from underrepresented groups, we will cover four specific areas:

- Vietnam experience-related measures
- Relevant confounders and covariates
- Data acquisition methods for veterans
- Issues of non-response and recall
- Generalizability.

RESEARCH DESIGN

Overview

The CU-AL cohort for the initial survey includes 6810 veterans, 2858 of whom served in Vietnam out of a population of 12,500 randomly selected American Legionnaires. The current study will re-survey all 12,500 veterans including those 6810 who participated in the 1984 survey and those Legionnaires who were identified as Vietnam Era veterans but did not return a questionnaire (original non-respondents). In addition, random samples of 100 Black and Hispanic and 50 female veterans who served in Vietnam will be obtained from outreach to other Departments within The American Legion (e.g., those located in Puerto Rico), and to other veterans' groups, such as the Women Vietnam Veterans Memorial Committee and the Vietnam Veterans of America. The Stellmans have had a long and fruitful working relationship with Vietnam Veterans of America and such outreach should be readily accomplished. These latter samples will serve as the study's key comparison groups for validating existing measures on military service and experiences.

Revisiting and Refining the Survey Instrument and Standardized Measures

The survey questionnaire used in the 1984 CU-AL study included sections on sociodemographic background, military service, combat, herbicide exposure, service locations in Southeast Asia, health-related behaviors such as smoking and alcohol consumption, and personal and family medical history. Each of these variables must be taken into account in any epidemiological study of veterans exposure to herbicides and related health outcomes.

The plan for refining the survey questionnaire, standardized measures and corresponding data collection procedures contains four key elements:

1. Collecting and reviewing existing instruments from other investigators and published research;

2. Conducting qualitative fieldwork and focus group discussions with target audiences of veterans to identify key issues and appropriate language;

3. Comparing reliabilities (Cronbach alpha) of scale items such as social adjustment, behaviorial, PERI and PTSD component scales with reliabilities measured in 1984.

4. Conducting reviews of instruments by members of the Advisory Committee, Legion field staff and members of the veteran community;

5. Pilot testing initial versions of the revised instruments and data collection procedures to ensure they are comprehensive and practical and yield reliable and valid data.

In refining empirical measures for each domain of interest, we will be guided by the following criteria: established reliability and validity for use with Vietnam-era veterans; congruence with a self-report format or a telephone interview; and efficiency to reduce respondent burden.

Conducting the Veteran Survey

The Sample

Our objective is to complete interviews with 70 percent of the eligible sample. Initially, the sample will include 12,650 Vietnam-era veterans (12,500 Legionnaires from the 1984 sample and 100 Black and Hispanic veterans and 50 female veterans). The entire list of sampled Legionnaires will be matched with updated mailing lists from the American Legion. It is estimated the Legion will have current addresses and telephone numbers for 50 percent of the sample (6250 veterans). The remaining list will then be reviewed by the Equifax Corporation, an organization specializing in providing current mailing addresses and telephone numbers. Equifax estimates they will be able to locate between 50 - 80 percent of the list. We will "guesstimate" at 60 percent (3750 Legionnaires). To locate the 2500 Legionnaires remaining in the sample, we will reinstate the American Legion Volunteer Groups employed in the 1984 study and use networking techniques to answer the following questions. Do you know where these people are? Are they still alive? If not earlier respondents, did they serve in Vietnam? We estimate that Legion networking efforts will locate 50 percent of the missing veterans (1250 Legionnaires). The additional samples of Black, Hispanic and women veterans will be drawn from current organizational files so up-to-date addresses and telephone numbers will be available (150 veterans). These efforts will yield an eligible sample of 11,400 Vietnam-era veterans (6250 Legion, 3750 Equifax, 1250 volunteers and 150 new sample).

To help increase cooperation, prior to any effort to collect data, the study will be publicized in American Legion publications and through a Legion networking effort. All sampled individuals will also be sent a cover letter. This letter will be written on Legion

stationery and sent first class mail in a Legion envelope. It should briefly explain the nature of the study, the importance of cooperating, and it should also alert the individual to a mailing or a call by a representative of the Legion or Columbia University School of Public Health.

Data Collection

Although our primary method for data collection will be self-administered questionnaires, in order to ensure that the desired response rate is achieved, we are proposing to use telephone interviews as well. Telephone interviews will be implemented in stages as the need arises.

Our objective is to complete surveys with 70 percent of the eligible sample of 11,400 veterans to yield a cohort of 7980 respondents. The 1984 survey achieved a response rate of 59 percent from a mailed survey. Given the amount of time that has elapsed from the initial study, for planning purposes, we shall assume that approximately 45 percent of the eligible sample will complete a self-administered questionnaire, that is, 5130 (11,400 x .45 = 5130) will complete a mailed survey. In order to complete the proposed 7980 interviews, 2850 telephone interviews need to be conducted with the remaining sample.

The first stage of our data collection design calls for attempting to complete by mail 5130 interviews. A self-administered mail-back version of the questionnaire will be posted to all eligible veterans along with a cover letter and a prepaid pre-addressed return envelope. Our approach to the mail survey will be based on the systematic set of follow-up procedures for non-respondents developed by Dillman (Dillman, 1978): (1) send a postcard follow-up reminder exactly one week after the first mailing; (2) three weeks after the first mailing, send a second cover letter and questionnaire to all those who have not yet responded; and finally, (3) seven weeks after the first mailing, send by priority mail a second cover letter complete with the earlier cover letter and a replacement questionnaire. Additionally, a check of $5 will be included in the third mailing as an incentive for the veterans to participate.

The revised questionnaire will also provide for the veteran to grant permission to obtain military records from the National Personnel Records Center, which we will do. These records are essential for Project 3.

The second stage of our data collection calls for completing by telephone interview the remaining 2850 surveys using random samples drawn from a list of veterans who did not complete the mail survey. Telephone interviews will take 15 minutes and be conducted by professional interviewers from the project staff. All interviewers will be experienced, fully-trained and closely supervised by the project director and interviewer trainer.

Data Processing

As mentioned previously, project staff will refine the survey instrument to be administered in this study. The original questionnaire included highly reliable measures/items for most of the variables in question, subsequent research and changes in the nature of the population demand that we repeat our earlier procedures of interviews

with Legion service represents and small focus groups of veterans. The final design of the questionnaire will include formatting the veteran survey for use as a self-administered questionnaire and as a telephone survey instrument. We will again pre-test the questionnaire by mail and for ease of response and clarity on a group of veterans in the South Dakota department of The Legion. After completion of the questionnaire, we will invite the respondents to a meeting to discuss the questionnaire, which we will have analyzed in advance to calculate reliabilities and to see whether any "problems" are apparent. The questionnaire will be revised, based on these pre-test results.

When a final draft of the questionnaire is available, the survey instrument and data collection procedures will be pre-tested on 200 eligible veterans: 100 self-administered questionnaires and 100 telephone interviews. The scheduling of the pretests will permit us to make any necessary revisions between pretests. The pretest data will also be used to re-examine the internal consistency of the measures included in the questionnaire. Additionally, responses to the pretest questionnaires will provide an opportunity to develop numerical codes for the open-ended questions included in the survey instrument.

Procedures for coding, entering and cleaning data that were established for the 1984 study will be updated for the current survey. All closed-ended questions will be pre-coded and, as noted, codes developed for open-ended questions.

The estimated 5130 self-administered questionnaires will be coded and edited by project staff and sent to the American Legion where specially trained key entry staff will use double-entry methods to process the data and edit the surveys.

When a clean data file is prepared, project staff will engage in two major types of error checking: (1) range checking and (2) contingency checking. Preliminary analysis will then be undertaken to gain familiarity with the data and to initiate scaling procedures and data reduction. Frequency distributions and descriptive statistics will be generated for all variable levels of missing data will be examined, procedures for handling missing data will be developed, and scales will be constructed for major study concepts. Internal consistency will be estimated by calculating Cronbach's alpha coefficient.

Data Analysis

Data analysis will focus on four groups of key variables: socioeconomic status (SES), combat exposure, herbicide exposure self-report, and a psychological variable analogous to a PTSD score. [Military history is not included here since it is the main focus of Project 3.] Two SES variables to be used are education and income level. Combat will be assessed by a validated scale used in our previous studies. A herbicide exposure scale is derived from a series of experiential questions, and the PTSD score is also obtained from a set of validated questions. These variable sets have been chosen because they are fundamental to health studies (SES), and are also unique to the veteran experience (combat, military herbicide use), or can affect response rates and data quality in any context (PTSD).

For continuous variables, comparisons between different sub-groups will be done by t-tests. Responses from the same veterans at two different times will be examined by paired t-tests. Where several simultaneous main effects are of interest analysis of variance will be used. Analyses will address the following questions:

a) How are these variables related to participation in the study at the two different times? This is a complex question, but one whose answer will help guide future studies by providing insight into who is more and who less likely to participate, and how those differences may be related to critical variables that are directly related to health outcomes.

Specifically, do subjects who provided both T1 and T2 interviews from participants at T1 but not T2 (hard to find now), compared with T2 but not T1 (original non-respondent who might have been found with more effort)?

b) Are response differences between T1 and T2 for the four variable groups related to exposure, as measured by EOI scores? That is, is herbicide exposure related to response itself? This could prove important in identifying for future studies potential selection bias directly related to exposures.

c) For those who responded at T2, do responses to these questions differ according to mode of interview (telephone vs. mail questionnaire)? This also can aid our understanding of the effect of survey methodology on possible outcomes.

d) Is there evidence that particular variables are confounders or covariates that must be taken into consideration in future epidemiological studies? That is, is the association between herbicides and health effects affected by these variables. We will examine the extent to which these etiological variables act as confounders of the associations between herbicide exposure (expressed either as an EOI or derived from the questionnaire) and health outcomes reported in the questionnaire. It is important to note here that we are not interested in the health outcomes observed, per se, (this is the subject of future epidemiological research). However, understanding this confounding is essential to planning such studies.

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PROJECT 3: Evaluation and Standardization of Military Personnel Records for Epidemiological Studies

PROJECT 3

SPECIFIC AIMS

Military personnel records contain information that will be essential to most epidemiological studies that involve exposure assessment. Few epidemiological researchers, however, are familiar with the existing records system and would be unlikely to know what records to request or how to utilize them. We will draw on our experience and familiarity with these records systems to develop a set of tools that can be used by researchers outside the military to obtain data essential for such studies. Our specific aims are:

1. To identify a standard set of military personnel records that comprise a minimum requirement for epidemiological studies of herbicide- and combat-related health effects

2. To develop and test a preliminary abstraction form to capture data needed for determination of an individual's military units, military occupations, and other service-related data relevant to herbicide exposure assessment

3. To evaluate these records for consistency, accuracy, and relevance to assessment of herbicide exposure by (a) obtaining sets of records for a sample of Vietnam veterans and comparing abstracted data with information gathered directly from the veteran via questionnaire; (b) identifying those veterans for whom there is serious discordance between military records and questionnaire with respect to military units, occupations, and other exposure-related items; and (c) conducting in-depth interviews with the discordant veterans, utilizing specially trained interviewers with military knowledge, to ascertain sources of discordance

4. Based on results of Aim 3, to modify and finalize the abstraction forms

5. To develop a guide to obtaining and abstracting military records for use in epidemiological research

Background

The United States armed services archive the military records, including medical records, of all personnel who are no longer on active duty. These records are kept in the National Personnel Records Center in St. Louis. Each branch of the service maintains different records and the records kept on any individual vary. The Principal Investigator and co-workers have had extensive experience in dealing with military personnel records in their work in support of the exposure evaluation of claimants to the AOVPP, during which time they examined at least 75,000 personnel records. Based on this experience, it was determined that the records listed in Table 4 were found to be the most useful sources of information that pertain to evaluation of potential exposure to herbicides during service in Southeast Asia.

The records listed in Table 4 are not consistent in their presentation of information. Indeed, during the course of the Vietnam War several of these forms underwent format changes so that equivalent data are not found in the same parts of all forms. For example, the Army Personnel Qualification Records underwent two transformations and there is a

separate form for officers during the early years of the War. The physical layout of the forms differ. An additional complication is that not all records are complete and often the military record researcher must undertake a mini-detection effort, piecing together corroborative bits of evidence from several different sources and forms in order to obtain a more complete picture of the exact nature of a veteran's military history.

Another reality is that these military personnel records were often not filled out at a leisurely pace in a location conducive to perfect record keeping practices. Inconsistencies are not uncommon, files may be handwritten and sometimes illegible. Further, there are multiple ways of referring to the same unit, so that interpretation of the records requires knowledge and experience. This project is intended to assist future researchers in making optimum use of available data in spite of these potential difficulties.

Although details about particular military experiences and exposures may be difficult to derive from the personnel record, in some instances where the study participant is deceased, unlocatable or otherwise unable to submit to questioning, either by mailed survey or interview, the personnel record may be the only reliable source of military experience data. For this reason it is extremely important for there to be a methodological appraisal of the adequacy/inadequacy of this data source. We will therefore evaluate the abstraction process for consistency, accuracy, and relevance. We will also develop a guide to the military personnel records and to develop abstraction forms.

Personnel vs. Unit Records.

This Project is concerned chiefly with personnel records, that is, those pertaining to individual veterans. In contrast, Project 1 dealt with records pertaining to military units. Examination of both types of records may be essential to construction of exposure histories for individuals.

Military Medical Records

Medical records are not considered in this proposal. Although veterans' personnel files do contain some health information, it is not generally useful for epidemiological research. An individual's personnel file contains only Field Medical Records which include primarily sick call entries with diagnoses and treatments. Hospitalizations would have been recorded in the Clinical Records of each hospital. To access this information the name of the hospital and the approximate dates of admission would be needed. This information might be found in the Field Medical Records from the time of the hospital admission, but this type of entry was not standard. Technically, a field medical form should have been completed for the Personnel file for each hospitalization, but these forms often did not find their way into the files. In summary, there is little standardized documentation of major illnesses or injuries that can be easily accessed using the material found the personnel files. If the veteran or survivor cannot provide the hospitalization information, there would be few usable medical records available.

Methods

This project will proceed in three stages:

- Forms development
- Discordance substudy
- Forms revision and guidebook

Stage 1: Forms development

We will work closely with military records experts to select forms that provide data on military units, occupations, and other exposure-related experiences, and create a uniform abstraction form on which these data can be recorded regardless of the specific source documents. Clerical personnel will be given drafts of the abstraction forms and sample source records representing each type listed in Table 4. They will learn how to "read" all of the standard forms, and will be trained in determination of UIC and MOS codes. Inter-user tests of reliability will be computed for key items, such as UIC codes, using kappa statistics. We will attempt to learn why any specific items exhibit poor concordance and, if indicated, modify the forms or instructions.

Stage 2: Discordance substudy

We will identify all the respondents from the American Legion cohort and outreach groups (Project 2) from whom completed questionnaires have been returned. Each respondent to the survey carried out in Project 2 will have been asked to sign a release form permitting us to have access to their military records. We will request the records of all respondents who served in Vietnam (approximately 4,000) and 500 selected from the controls. The National Personnel Records Center has indicated that this will be possible but that we must hire on-site personnel to retrieve and photocopy the particular records from the pulled file. They will be abstracted using the forms created in Stage 1 above. The records will be reviewed and coded blind to the results of the survey questionnaire in order to extract units and dates of service and any other information on service that is relevant to potential exposure to herbicides or to heavy combat situations. These data will be computerized and merged with the questionnaire data. Data items from both sources will be compared to determine the extent of agreement or discordance. Veterans for whom there is a high level of discordance (to be determined in initial sample) will be identified, their records re-examined and their names and telephone numbers recorded.

Specially recruited and trained interviewers with knowledge of military records will then contact each of the discordant veterans and conduct a carefully structured telephone interview designed to determine the source of discordance. The interviewers will be carefully monitored by senior Project personnel.

Analysis of data

Our data analysis will focus on consistency and accuracy.

- We will examine whether consistent results are obtained from different types of military records
- Accuracy of veteran self-report will be evaluated by comparison of questionnaire data against data in the military records

Other important analyses

In addition to consistency and accuracy, the actual relevance of data gleaned from records will be examined by comparing surrogate exposure measures commonly reported in the literature with the exposure opportunity indices calculated from the military service unit and from the veteran's self-report of exposure. Review of the surrogate measures listed in Table 1 of this proposal shows that several published studies have used broad military classifications such as Branch of Service, MOS, tactical corps (I Corps, II Corps,

III Corps, IV Corps) as surrogates for exposure. We will compare the MOS (military occupational specialty) with the tasks which the veterans reports having carried out while in Vietnam. We are also interested in corroborating the combat experience of the veterans since, as discussed in Project 2, exposure to combat is an extremely important factor to be taken into consideration.

Stage 3: Forms revision and guidebook

Based on results of the discordance substudy and in-depth discussions with the interviewers, we will revise the abstraction forms as deemed appropriate, and develop a finalized guidebook to their use.

FINAL PRODUCT

The end product of PROJECTS 2 & 3 will be a set of systematic, reliable, accurate, and well documented procedures for reconstructing the military experience relevant to the health outcomes of interest.

Table 4

Military Personnel Records Potentially Useful for Epidemiological Research on Herbicides and Health

Branch of Service and Form type	Type of Form	Information Available
ARMY		
DA Form 20/24/66/2-1	Personnel Qualification Records	Dates and location of foreign service; units of assignment with "in" and "out" dates; MOS (duty code); description of primary duty; awards and medals
Special Orders		Dates of transfer from unit to unit; dates of awards, medals, promotions, etc. and unit assignment at the time
MARINES		
NAVMC 118(3)	Record of Service	Units of assignment with "in" and "out" dates; primary duties
NAVMC 118(9)	Combat History/Expeditions	Dates "in" and "out" of Vietnam operations; awards with dates and units of assignment at the time
NAVMC 118(17)	Sea and Air Travel Embarkation Slips	Dates of transfer to and from Vietnam
Unit Diaries		Listings of veterans assigned to the specific units for specific dates; unit movements
AIR FORCE		
AF FORM 7/11	Airman and Officer Military Records	Dates and locations of foreign service; units of assignment with "in" and "out" dates; Air Force Base assignments, duty codes, awards and medals
Performance Reports		Unit of assignment with "in" "out" dates; Air Force Base of assignment, description of duties
NAVY		
NAVPERS 601(12)	Transfers and receipts	Dates of transfer between units and ships
NAVPERS 601(13)	Administrative remarks	Units and/or ships of assignments, dates, some ship activities
ALL BRANCHES		
DD214	Report of Transfer or Discharge	Dates of service; dates of foreign service; last unit of service and awards
Unit Histories (Army, AF, Marines)		Unit activities and movements
Ships' Histories (Navy, Marines)		Ship activities and movements

PROJECT 4: Biomarkers of TCDD Exposure in Vietnam Veterans

Overview

The presence of TCDD in adipose tissue and serum can be a direct measure of exposure to dioxin-contaminated herbicides. However, in the case of Vietnam veterans, and as with most retrospectively collected biomarkers used to estimate exposure in epidemiological or clinical research, there are serious methodological problems attached to its use. The primary problem is, of course, the long period of time that has elapsed since the actual exposure may have occurred. Negative findings are not unambiguously interpretable: the absence of TCDD can signify either no exposure or the metabolic disappearance of TCDD that had once been present. When TCDD is detected, there is insufficient knowledge about the kinetics of TCDD, metabolism, and storage and individual variation to allow extrapolation of current levels of TCDD to past exposures.

Further, the determination of TCDD is an extremely costly and time-consuming laboratory procedure. A comparative handful of laboratories around the world have the capability of running the required high resolution gas chromatography/ high resolution mass spectroscopy (HRGC/HRMS) procedures. Much time and money are expended on analysis of negative (no TCDD present) samples.

Despite these methodological barriers, some useful data might be still be gathered. For example, in re-examining CDC data on TCDD levels in serum taken from 696 Vietnam veterans, Stellman and Stellman found that by stratifying the sample, exposure opportunity index scores (EOIs) were significantly correlated with TCDD levels found in those veterans whose EOIs were in the upper quartile and decile. (See summary of calculations in the accompanying box.) The levels observed by the CDC in 1986-7, in the most highly exposed veterans (up to 45 ppt) should still yield elevated TCDD in 1998 when a half-life of 7 years is applied. Thus, if sampling is restricted to veterans with the highest EOIs, it might still be possible to find and employ TCDD levels as one kind of validation of the historical reconstruction methods proposed here.

Further, in the past several years a recombinant cell bioassay system that responds to TCDD and related chemicals with the induction of firefly luciferase expression has been developed [Garrison & Tullis et al, 1996]. Such a system appears to represent a rapid screening assay. A sensitive, specific, rapid and relatively inexpensive screening procedure could potentially make more routine the use of TCDD assays in epidemiological studies since a screening test could be used to determine which samples were "positive" and only these would then be subjected to the costly (and difficult to schedule) HRGC/HRMS methods.

The toxicity of polychlorinated diaromatic hydrocarbons (PCDH), such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is mediated by the Ah receptor (AhR), a ligand-dependent transcription factor which activates gene expression. Xenobiotic Detection Systems has developed a mechanistically based recombinant cell bioassay system called CALUX that uses induction of firefly luciferase as a reporter to measure the concentration of dioxin-like toxicants (TEFs) in biological samples. The bioassay should provide a rapid and quantitative screening method for analysis of the concentration of dioxin-like toxicants (TEFs) in serum.

CORRELATION OF SERUM TCDD IN GROUND COMBAT TROOPS WITH EXPOSURE OPPORTUNITY INDICES

In 1988 the CDC reported serum TCDD levels measured in 646 ground combat troops, using blood drawn in 1986-87(CDC, 1988). Correlation coefficients (ρ) between TCDD and each of six variant exposure opportunity indices (including one similar to our E3 score) were all below 0.06 in magnitude, except for one (ρ =0.13), the sole ρ that was statistically significant. This was interpreted as a failure to validate EOIs as surrogates for TCDD measurements.

In reviewing these data, we noted that the number of men with substantial exposures was rather small. The distribution of "hits" (present within 2km and 6 days of any spray) among the study participants was as follows:

No. "hits"	No. men exposed	(%)
0	298	(46.1)
< 5	157	(24.3)
5+	191	(29.6)
Total	646	

In other words, nearly one-half were not near any spray. In an attempt to increase the sensitivity of the study and compensate for the small number of potentially "exposed" study members, two re-analyses were carried out by us: one was restricted to men whose serum TCDD levels placed them in the upper quartile and the second further restricted to men in the upper decile. These restrictions were based on the reasoning that men with the lowest TCDD levels could either have low levels because they were not exposed, or because they had already excreted or metabolized the TCDD which they had absorbed, or because the military records did not accurately predict their exposure status. Thus, because of these varied possibilities, the low TCDD scorers represented a poorly defined group for study and hence maximized the possible misclassification problem. On the other hand, men in the upper quartile or decile of exposure had obviously not exceeded the time required for TCDD to decay to background, (although their metabolic rates may still have differed within the group) and therefore represented a group with a smaller likelihood of misclassification. The resulting ρ's are shown below for the original cohort and the two restricted subgroups:

		EXPOSURE OPPORTUNITY SCORE				
	No. subjects	a	b	c	d	e
f						
All 646		-0.05	0.06	0.04	-0.04	0.13***
0.06						
Upper 25% (N=173)	-0.06	0.22**	0.19*	0.00	0.38***	0.24**
Upper 10% (N=65)	-0.14	0.32*	0.27*	-0.07	0.47***	0.29*

Thus, CDC data show that, at least for serum drawn before 1988, serum dioxin was significantly correlated with several EOI measures.

Reference: Centers for Disease Control, Serum 2,3,7,8-tetrachlordibenzo-p-dioxin levels in US Army Vietnam-era veterans. JAMA 1988; 260:1249-1254.

Thus, despite the fact that caution must be used in interpreting the results of biomarker tests of Vietnam veterans for herbicide exposure, a well-designed laboratory study could yield valuable data for substantiating the credibility of herbicide spraying data and of the exposure opportunity index based on historical reconstruction.

A comparison between the CALUX results and the HRGC/HRMS method, carried out on split-samples taken from veterans with high EOIs (and appropriate controls) will provide a reliable test of the utility of this method as a screen in future epidemiological studies. The project proposed here will also permit us to determine whether the EOIs that we calculate predict the TEQ scores obtained from the CALUX screen.

Overall objectives

The overall objectives of Project 4 are to determine: 1) whether the calculated exposure opportunity index can successfully predict the observed TCDD levels in heavily exposed Vietnam veterans; 2) whether a recently developed cellular assay can serve as a screening test for such exposure; 3) whether the use of TCDD assays or a rapid screen substitute is a practical methodology to be included in future epidemiological studies of Vietnam veterans.

Midwest Research Institute's (MRI) objectives

The objectives are to provide analytical support services for the determination of 2,3,7,8-TCDD and other 2,3,7,8-substituted PCDDs/PCDFs in human tissues of South Vietnamese nationals (adipose in IARC archive) and Vietnam-era veterans and controls (blood serum), based on the study design described in this proposal. MRI is responsible for 1) providing this technical support through the use of validated and published analytical protocols; 2) integrating a stringent QA/QC program that will provide consistency in measurement(s) across the proposed study period and allow comparison to other existing databases and assess comparability of methodology between HRGC/HRMS and the CALUX bioassay; and 3) developing essential quality control pools (human adipose tissue and blood serum samples) of sufficient volume and characterization to demonstrate comparability of the results throughout the study.

Xenobiotic Detection Systems' (XDS) objectives

The major objective of the XDS sub-project is to ascertain whether the CALUX bioassay achieves sufficient sensitivity and specificity (i.e. elevated TEFs in serum correspond to congener specific results of HRGC/HRMS analysis with acceptable false positive and false negative rates) regardless of the concentration and distribution of congeners present. A second objective is to determine whether the body burden of TEFs itself could reliably be used as an exposure measure in future human health effects studies.

METHOD

The analysis of samples has been planned to begin after a substantial proportion of the interviews described in Project 2 have been carried out and after an extensive amount of work has already been completed on Project 1, the sensitivity analysis of the MUD-V database used for the calculation of EOIs. Based on the data obtained in the survey, every Vietnam veteran who scores "high" or who held a

military occupation that involved herbicide exposures will be invited to participate in the serum studies. Based on the findings of our earlier studies of this group, we estimate that approximately 500 veterans will fall into this category. An additional 100 veterans will be selected to participate and will be drawn from low/no, and medium EOI levels. Samples will be collected and analyzed by HRGC/HRMS and CALUX methods described below. The blood samples will be drawn by a competent technician who meets the requirements set forth by each of the states in which the participants live.

A short questionnaire will be developed, tested and administered to the participants by the technician in order to ascertain whether they have worked with or used herbicides or other chemicals which might lead to an expected elevation in the blood TCDD levels. Persons with known occupational exposures will be excluded.

Technical Approach for the Analysis of Human Tissues (adipose and blood serum) for 2,3,7,8-substituted PCDDs and PCDFs.

Sample Collection Protocol

The human adipose tissue samples (200) will be provided to MRI for analysis from the IARC specimen repository. MRI's procedures require a minimum of 1 g of human adipose tissue or 50 mL of serum to achieve detection limits of 0.5 pg/g (2,3,7,8-TCDD equivalents) lipid adjusted concentrations.

The blood serum samples designated for analysis consist of approximately 600 samples to be collected in Project 4 below. The specimens will be transferred frozen to MRI's laboratory and will be maintained at -10 C until analysis procedures are initiated.

The proposed analytical methodologies can accommodate blood serum volumes of 25 to 50 mL. MRI prefers to work with sample sizes of 50 mL. The screening procedure, the CALUX method, requires 2 to 5 mL of serum. Hence a total of 65 mL of sample are recommended to complete the objectives for the study.

Qualified phlebotomists will obtain a 65 mL sample of blood serum in accordance with standard blood collection procedures. MRI is highly experienced in blood collection

A copy of MRI's general blood collection protocol is available for review. The whole blood will be centrifuged to separate the blood serum. The blood serum will be transferred to Teflon bottles and frozen. The samples will be shipped to MRI via overnight mail. MRI will be responsible for splitting the samples for the HRGC/HRMS analysis and the CALUX method. MRI will ship serum samples and quality control samples for the CALUX method to Xenobiotic Detection Systems (XDS) via overnight delivery.

HRGC/HRMS Analytical Approach

The analytical methods proposed by MRI are based on high resolution gas chromatography/high resolution mass spectrometry (HRGC/HRMS). The sample preparation procedures were originally developed for the analysis of adipose tissue samples from the EPA National Human Adipose Tissue Survey (NHATS) and for the analysis of blood serum samples for the Michigan Agent Orange study. MRI's analytical protocols and proficiency in the analysis of the 2,3,7,8- substituted PCDDs and PCDFs

has been demonstrated through successful performance under the World Heath Organization's round robin analysis of human serum samples.

Quality Assurance/Quality Control (QA/QC)

The QA/QC program will consist of several elements:

•established sample collection and analysis protocols

•blind and double-blind coding of specimens and associated QC samples

•specified data quality objectives and performance requirements for calibration, recovery, accuracy and precision

•incorporation of well characterized adipose tissue and blood serum pools to provide both intra- and inter-laboratory performance criteria.

Analysis of results

We will examine the relationship between the computed EOI scores for those who contributed serum and the measured serum levels of dioxin and dibenzofuran congeners, using several complementary statistical approaches:

a) Graphical displays using scatterplots of serum levels vs. EOI scores, and box plots of serum levels vs. EOI tertiles to visualize correlations

b) Statistical correlation analyses, using Spearman correlation coefficients for bivariate correlations between EOI scores and serum levels (log-transformed if distributions appear to be log-normal) of total PCDDs/PCDFs or individual congeners

c) We will examine the sensitivity of the serum-EOI correlations to selection of alternative EOI models and study the extent to which the rank orders of EOI scores differ among diverse models, using nonlinear structural modeling methods (Carroll et al. 1994)

CALUX

Background

Polychlorinated diaromatic hydrocarbons (PCDHs) are a diverse group of widespread environmental contaminants, which include polychlorinated biphenyls (PCBs), dibenzofurans (PCDFs), dibenzo-*p*-dioxins (PCDDs) as well as many other subclasses of PCDHs [Giesy & Ludwig, 1994a]. Exposure to and bioaccumulation of PCDHs has been observed to produce a variety of species- and tissue-specific effects, such as tumor promotion, lethality, birth defects, hepatotoxicity, immunotoxicity, dermal toxicity, alterations in endocrine homeostasis and induction of numerous enzymes, including that of cytochrome P4501A1 [Giesy & Ludwig, 1994b][Poland & Knutson, 1982][Safe, 1994][DeVito & Birnbaum, 1994][Luster & Faith et al, 1979][Lucier & Tritscher et al, 1991][Clark & Blank et al, 1991][Taylor & Lucier et al, 1992][Clark & Tritscher et al, 1992a]. PCDHs differ in their biological and toxic potency so accurate prediction of the biological/toxic activity of complex PCDH mixtures using chemical specific methods of HRGC/HRMS is difficult. Toxicological potency of a complex mixture of PCDH chemicals is assessed by the toxic equivalent factor (TEF) approach in which the concentration of the individual compounds present in the mixture are multiplied by their

specific TEF and the sum of the values is expressed as TCDD toxic equivalents (TEQs) [Safe, 1994][Safe, 1990][Ahlborg & Brouwer, 1992)].

Numerous biochemical and genetic studies of the mechanism of action of PCDHs have utilized 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin), the prototypical and most potent PCDH. These studies have focused on the induction of microsomal cytochrome P4501A1 and its associated monooxygenase activities [Poland & Knutson, 1982][Whitlock, 1993] [Hankinson, 1995]. This work has revealed that induction of P4501A1 is mediated by a soluble intracellular protein, the Ah receptor (AhR), to which TCDD and related PCDHs bind with high affinity [Poland & Knutson, 1982][Safe, 1994][Whitlock, 1993][Safe, 1986]. After ligand (TCDD) binding, the receptor translocates into the nucleus [Hord & Perdew, 1994][Pollenz & Sattler et al, 1994] where it dimerizes with a second protein (the Ah receptor nuclear translocator (ARNT)) and the heteromeric AhR complex binds with high affinity to a specific DNA sequence, the dioxin responsive element (DRE). The binding of the TCDD:AhR complex to the DRE stimulates transcriptional activation of the adjacent responsive gene (i.e., Cyp1A1) [Whitlock, 1993][Denison & Fisher et al, 1988][Denison & Fisher et al, 1988b][Lusska & Shen et al, 1993][Yao & Denison, 1992]. In addition to the correlation between AhR-ligand binding and P4501A1 induction, quantitative structure-activity relationship (QSAR) studies have revealed a good correlation between the ability of PCDHs to bind to the AhR and their toxic potency, supporting a role for the AhR in mediating the toxicity of these chemicals [Poland & Knutson, 1982][Safe, 1994][Safe, 1986][Goldstein & Safe, 1989].

The above results, coupled with the ability of the AhR to act as a ligand-dependent transcription factor, supports the hypothesis that many of the toxic/biological effects of these chemicals are mediated by the AhR and result from differential regulation of gene expression. Consequently, the induction of P4501A1 and its monooxygenase activities (7-ethoxyresorufin-O-deethylase (EROD) or aryl hydrocarbon hydroxylase (AHH)) has been one response that has been used as a bioassay to evaluate the relative biological/toxicological potency of PCDHs and complex mixtures containing these chemicals [Giesy & Ludwig, 1994b][Safe, 1994][Safe, 1990][Casterline & Bradlaw et al, 1983][Tillitt & Giesy et al, 1989][Tillitt & Ankley et al, 1991]. The results of these studies have revealed a strong correlation between the induction of EROD/AHH activity and the toxicity of various PCDH congeners and PCDH-containing mixtures [Giesy & Ludwig, 1994b][Safe, 1994][Safe, 1990][Mason & Sawyer et al, 1985]. However, the use of EROD activity as a bioassay for PCDHs is complicated by the fact that many inducing chemicals are also substrates for the enzyme. Taking advantage of existing knowledge of the molecular mechanism of AhR action (the ability of DREs to confer TCDD-responsiveness upon an adjacent promoter and gene [Whitlock, 1993][Denison & Yao, 1991]), combined with the availability of several sensitive reporter genes [Alam & Cook, 1990], a recombinant PCDH-inducible expression vector was constructed which contain an easily measurable and extremely sensitive reporter gene (firefly luciferase) under PCDH-inducible control of four DREs. Luciferase activity is not adversely affected by PCDHs, is more sensitive, rapid and amenable to automation than the EROD assay.

Preliminary Studies:

Mouse hepatoma (Hepa1c1c7) cells stably transfected with this vector have produced a novel cell line (H1L1.1) in which PCDH-inducible expression of luciferase

occurs in a dose-, time-, and chemically specific- Ah receptor (AhR)-dependent manner (Figure 3). These recombinant cell bioassays are quick and sensitive (with minimal detection limits for TCDD of 0.001 nM (0.3 pg TCDD/assay)) and may be used for detection and relative quantitation of a wide variety of PCDHs and PCDH-like chemicals in samples and sample extracts [Garrison & Tullis et al, 1996].

This recombinant cell bioassay system was originally developed to detect PCDHs in serum and the serum bioassay has been submitted to ICCVAM (Interagency Coordinating Committee on the Validation of Alternative Methods) for validation. The assay has also been adapted for additional sample matrices [Aarts & Cenijn, et al, 1996][Murk & Jonas, et al, 1996][Bovee & Hoogenboom, et al, 1996]. A synopsis of the development of the assay for serum samples follows:

This cell line is highly responsive to PCDHs and produced a very sensitive bioassay for PCDHs. In contrast to using P4501A1 EROD activity as a bioassay system where different chemicals induce EROD activity to different maximal values, each of the active chemicals in this bioassay induce luciferase activity to the same maximal value. This is due to the fact that the inducing chemicals can act as competitive substrates for P4501A1 but not for luciferase. This allows the luciferase-based bioassay to provide more accurate estimates of the potency of the inducing chemical(s) with our assay system. We have developed this assay so that it can be done in an automated microplate luminometer. The instrument automatically injects an aliquot of the assay substrate into each well and monitors the subsequent luminescence production. The assay can be useful for the direct measurement of TCDD and TEQ equivalents in unextracted serum if the concentrations of the toxicants are approximately 15 ppt on a lipid adjusted basis. Greater sensitivity is obtained with solvent extraction. Following solvent extraction, concentrations as low as 0.1 ppt are detectable, as is demonstrated in figure 4, which shows the differential sensitivity of the assay for TCDD in serum versus extracted and dissolved in DMSO.

This difference in sensitivity is presumably due to binding of the chemical by serum proteins. Use of serum samples directly in the bioassay will be appropriate only when estimating high blood levels of PCDHs. We have developed methods to concentrate the dioxins and dioxin-like compounds from serum samples and now have attained levels of detection of 5 fg of dioxin or dioxin equivalents in a sample after appropriate processing of the sample. This corresponds to a detection limit of 0.025 ppt if an initial sample size of 1 ml of serum is used for analysis

To make our assay useful for risk assessment we developed the capability to dissect the contribution of PCBs and dioxin/furan contaminants in a sample to the total TEQ. The basis of this separation is differential absorption and elution of the analytes on acidic alumina [Turner & DiPietro et al, 1994] [Albro & Corbett, 1977]. PCBs and dioxins/furans can be differentially eluted with varying mixtures of hexane and methylene chloride. We have used this method to evaluate the contribution of PCBs and dioxins / furans. Like HRGC/HRMS methods, it permits estimation of the contribution of these major classes of halogenated aromatic compounds to the total TEQ in complex mixtures.

Comparison of the HRGC/HRMS and CALUX methodologies

The data analysis goal for the CALUX sub-project is to establish the utility of the CALUX method as a screening tool for detection of PCDDs/PCDFs in serum. CALUX provides an estimate of TEQ, but not actual dioxin/furan concentrations; ideally, a low

CALUX TEQ should imply a low probability of detecting dioxins/furans via the more expensive high-resolution GC/MS assay. Thus, if proven effective, CALUX could be used to decide that the sample is from an unexposed individual, or whether an additional serum sample should proceed to GC/MS for a quantitative assay.

Effectiveness as a screen will be accomplished by comparing the CALUX results, expressed as dioxin/dibenzofuran TEQs, for serum samples collected from 600 Vietnam veterans interviewed in Project 2 with PCDD/PCDF assays obtained from the same split samples, analyzed by MRI using high-resolution GC/MS. The latter will be considered the reference ("gold standard"). We will compare TEQs reported from CALUX assays (TEQ-C) with TEQs calculated from the concentrations of congeners obtained with GC/MS (TEQ-HR). TEQs for the latter method will be computed as a weighted sum of concentrations of individual congeners, using as weights toxic equivalence factors reported in the literature. Each veteran's split serum sample will thus yield two numbers: TEQ-C from the CALUX screen and TEQ-HR from the reference GC/MS assay.

Sensitivity (true positives divided by true positives plus false negatives) and specificity (true negatives divided by true negatives plus false positives) will be computed as a function of a range of cutoff levels for TEQ-C. A cutoff is a specific TEQ that defines whether the veteran is declared "probably unexposed" or "probably exposed." For each possible cutoff, we are interested in determining the probability that the specific sample would have been above or below detection limits via GC/MS. Effectiveness of CALUX as a screening tool implies both high sensitivity and specificity. Establishment of a reliable cutpoint will make it possible in future epidemiological studies to decide rapidly and inexpensively which samples should be assayed with GC/MS. Since the majority of veterans are unexposed, this could result in very large cost savings.

We can also take advantage of the fact that CALUX results are quantitative (not just yes/no), by producing scatterplots of TEQ-C vs. TEQ-HR to visualize correlations, and computing correlation coefficients. This will be done using the full set of data, and separately in strata TEQ-HR, to assess how accurately CALUX predicts GC/MS results at different dioxin concentrations. We will examine whether the observed relationships are affected by log-transformation of the TEQ's. Large correlations could imply that CALUX can be used in studies that relate health effects to TEQs rather than congener levels.

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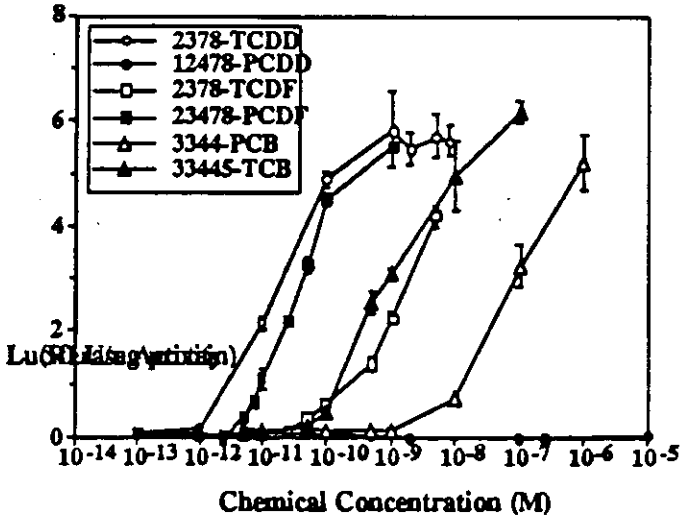


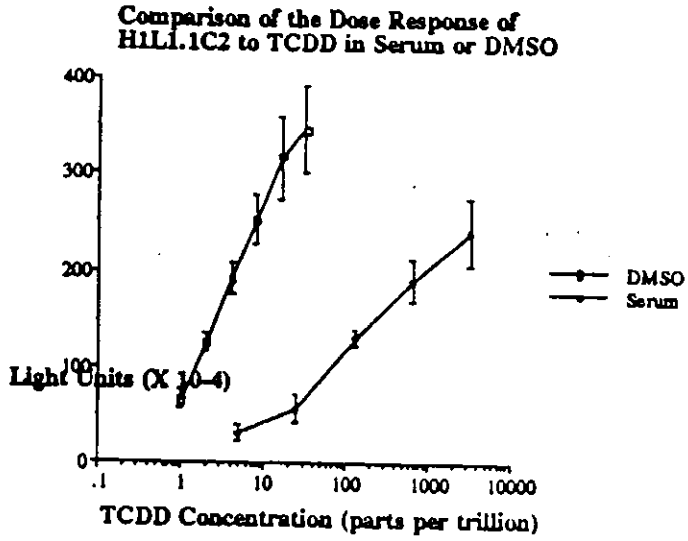


Figure 3. Effect of various PCDHs on luciferase activity in stably transfected mouse hepatoma cells. Cells were incubated with increasing concentrations of each chemical in DMSO and luciferase activity determined as described in text with the response being normalized to protein concentration per well.

Figure 4. Dose response curves for induction of luciferase in H1L1.1 cells incubated with the indicated concentration of TCDD dissolved in DMSO or serum. Cells were incubated with increasing concentrations of TCDD for 4 h and luciferase activity of cell lysates determined

PROJECT 5: Analysis of IARC Tissue Samples of Selected Vietnamese for dioxin and dibenzofuran levels in archived adipose tissue

BACKGROUND

One method of validation of data on the extent and location of herbicide spraying in Vietnam could be to compare results of biological monitoring for herbicide residues (specifically dioxins) of Vietnamese citizens, their lifetime residence and herbicide exposure histories and the HERBS tapes spray data. The rationale for such an undertaking could include the following:

1.Dioxins are persistent in the environment and are representative of spraying patterns.

2.Dioxins are persistent in adipose tissue.

3.Lifetime residential histories could be accurately obtained.

Each of these propositions is subject to potential methodological problems:

1. Herbicides and their dioxin contaminants are decomposed in the environment by the action of sunlight, bacteria, and metabolism via flora and fauna; they are also subject to environmental transport through soil and water runoff.

2. While dioxins and other organochlorine compounds persist in humans, they are eventually metabolized or otherwise mobilized and excreted at rates that may depend on body mass, nutritional status, and other variables.

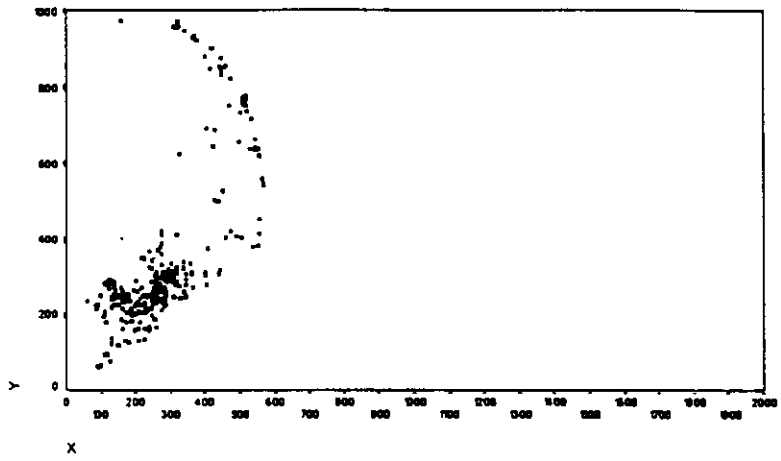
3. Persons may not be able to recall accurately their whereabouts during a time of war that occurred up to three decades prior to interview.

However, a limited number of studies by Schecter and colleagues (1994) have confirmed that levels of dioxins in the serum of South Vietnamese civilians up to two decades after the War were considerably elevated (TCDD: 3.4 to 28 ppt lipid basis) compared to serum of North Vietnamese residents (1.2 ppt), which were comparable to background levels in the U.S. The foregoing measurements were carried out using opportunistic samples of blood pooled from batches of fifty Vietnamese of unstated age and residential history. It is therefore difficult to link such observations quantitatively to exposure histories. Verger et al. (1994) using a modification of our published exposure estimation methodology (Stellman and Stellman 1986) found 11 of 27 Vietnamese subjects with elevated TCDD levels in adipose tissue, and only five with zero exposure. Verger et al. reported a significant correlation between the HERBS-file based exposure opportunity score and adipose TCDD. However, this sample is quite small and may not represent an adequate range of exposure histories.

A useful archive of adipose tissue taken from nearly 1,000 Vietnamese residents has recently been established by the International Agency for Research on Cancer (IARC) and will be available for this study. Figure 5 shows the geographical distribution of residence locations of the first 300 IARC study subjects. It is especially important to note that this distribution provides a good geographical representation throughout former South Vietnam, and clearly is not merely a sample from former Saigon. This study has the advantage of utilizing persons with potentially extensive residential exposure to the same herbicide applications as U.S. veterans. Furthermore, higher initial exposures make it more

likely that detectable levels still existed into the 1990s when the adipose tissue samples were obtained. A disadvantage is that there may have been unrecorded sources of subsequent exposure among this population and residential histories may be inaccurate.

FIGURE 5 - Locations of first 300 patients in IARC case-control study



APPROPRIATE SAMPLES AVAILABLE

For the present study, we propose to carry out dioxin/dibenzofuran assays on a limited number of carefully selected adipose samples from the IARC study, which is described below. Considerations of cost and efficiency dictate that the smallest possible number of samples be assayed, consistent with optimizing the likelihood of distinguishing low from high exposures. This will be accomplished by first estimating exposure opportunity for all subjects (a simple and inexpensive task once the exposure score refinement task is completed), and then selecting IARC study participants from low, medium, and high exposure strata.

The specific aim is to examine the relationship between a HERBS-file based herbicide exposure opportunity index (EOI) and current dioxin levels in the adipose tissue of Vietnamese.

Four steps are required to satisfy this aim:

1.Evaluate EOI for all available case-control study participants

2.Select a sub-sample of participants from several EOI strata

3.Obtain and assay their adipose tissue for dioxin and other chemical species of interest

4.Carry out the correlation analysis

BACKGROUND OF IARC TISSUE ARCHIVE

During 1993-1996 IARC carried out a case-control study of soft-tissue sarcoma and non-Hodgkin lymphoma in Ho Chi Minh City (formerly Saigon). The study was hospital-based, using the Oncology Centre of Ho Chi Minh City. This institution treats 5,000 in-patients per year, one-third of whom are residents of Ho Chi Minh City, and the remainder from elsewhere in Southern Vietnam. Diagnoses were confirmed with morphological and immunocytochemical techniques by laboratories in Paris and Bilbao.

Two sets of hospital controls were selected. The first control uses cancer patients diagnosed at the Oncology Centre with cancers not known or suspected to be linked to exposure to herbicides, pesticides, or other sources of dioxin. Excluded are Hodgkin's disease, respiratory tract and prostate cancers, thyroid and endocrine tumors, multiple myeloma, and the leukemias. The second set of controls comprises patients attending the Oncology Centre who do not have cancer. These include goiters, fibroadenomas, cutaneous lesions, epidermoid cysts, and non-malignant lymphoadenopathy. One control from each source is matched with each case for sex, age within five years, and current residence within or outside Ho Chi Minh City (to control for socio-economic and urban/rural status). No closer residence matching is used, in order to avoid overmatching on residential exposure. Use of Oncology Centre patients is an especially valuable feature because controls are generally comparable to cases with respect to nutritional factors like weight loss that could affect mobilization of dioxin in fat.

On cases, and control subjects undergoing surgery, informed consent was obtained for taking a specimen of ca. 2 g of adipose tissue during the operative intervention. All specimens were deep frozen and later transferred to the freezers at IARC in Lyon, France. Each patient also gave a residential history dating back at least to 1965 (first year of widespread herbicide use by US military), with all subsequent residence changes. A gazetteer of place names, by province, in southern Vietnam was prepared and the residential histories were keyed to the same UTM coordinate system utilized on the HERBS files. Thus, it is possible to carry out exposure assessments in a manner equivalent to our previously published method (Stellman & Stellman, 1986) and similar to the methods employed by Verger et al. (1994).

METHODS

Step 1. Evaluate Exposure Opportunity Index (EOI) for all available case-control study participants: The purpose of this step is to establish putative exposure strata from which adipose samples can be selected. We plan to carry out the selection procedure using the following procedure. Table 5 contains a residential data base for the first twenty subjects in the case-control study, with the ID number, the UTM coordinate, and the years of residence. Multiple locations and residence dates are given for several subjects (ID Nos. 0002, 0007, 0011, 0014).

Table 6 shows the results of carrying out an exposure analysis on these 20 people, using our previously published algorithm. We evaluate each residence separately, then sum multiple residences for each individual. Table 6 also gives the number of Agent Orange hits within 5, 10, and 15 km, as well as an integrated exposure measure (E3). It shows mostly importantly that even within this small sample there is a substantial range of exposure scores, so that there should be little difficulty in selecting a stratified sub-sample.

Step 2. Select a sub-sample of participants. We will obtain from IARC a complete list of study subjects for whom adipose tissue is available, and who lived at the same residence during 1965-1971 (the years of major herbicide spraying). The list will contain only ID Numbers, age, and residential history sufficient to calculate EOI scores. We will then create three exposure strata based upon tertiles of the distance- and time-weighted score (E3). All persons with valid integrated exposure scores will be categorized either as zero or non-zero exposure. Those with non-zero exposure will further categorized as falling above or below the median among those with non-zero scores. Twenty persons will be sampled at random from each of the three strata so defined, in such as way that ten fall above the median age and ten below. This will assure us of a sufficiently wide range of scores for comparison within strata with similar age distributions.

All selections will be made blind to the health status of the study subjects. Note that it does not matter whether samples are obtained from cases or controls, since the issue under study is simply exposure, and not epidemiological findings or health outcomes. It is advantageous to use such patients whether or not a link with the index cancers is proved. If no such link exists, then the patients in essence constitute a non-systematic sample of hospital patients, while if herbicide exposure is indeed responsible for their illnesses then high exposures should be over-represented in our sample, which will increase statistical power.

Step 3. Obtain and assay their adipose tissue for dioxin and other chemical species of interest. The tissue samples for each of the sixty people selected in Step 2 will be identified and sent to the collaborating laboratory. All assays will done by personnel blinded to the location and case-control status of the study subjects. Assays will be carried out for the total dioxins, total dibenzofurans, and congener species indicated in the literature as being frequently detected in human adipose tissue (1), as described in Project 4 above.

Step 4. Carry out the correlation analysis. The final step of this project will be to examine the relationship between the computed EOI scores and the dioxin/dibenzofuran levels in the adipose tissue of the study subjects. Several approaches will be used:

a) Graphical displays using scatterplots of tissue levels vs. EOI scores, and box plots of tissue levels vs. EOI tertiles to visualize correlations

b) Statistical correlation analyses: Spearman correlation coefficients for bivariate correlations between EOI scores and tissue levels (log-transformed if distributions appear to be log-normal) of total PCDDs/PCDFs or individual congeners

c) We will examine the sensitivity of the correlations to errors in the residential coordinates from which EOIs are derived, and the extent to

which rank orders of exposure scores differ among diverse models, using nonlinear structural modeling methods (Carroll 1994)

Statistical methods and sample size determination

To measure the association between the exposure opportunity index and selected dioxin adipose levels, Pearson's correlation coefficient (ρ) will be used. If necessary, the logarithmic transformation will be used to normalize exposure and/or dioxin levels. Prior to analysis, subjects will be classified as either Low, Medium or High exposure corresponding to tertile category based on the exposure index score. Within each of the three groups, correlations will be computed. In the table below, minimum detectable correlation ρ's for 80% power and 2- tailed alpha=0.05 for 50 to 100 observations given:

n	50	60	70	80	90	100
ρ	.38	.35	.33	.31	.29	.28

Verger et al. reported a statistically significant Spearman ρ of 0.44 (p=0.04) and Pearson ρ of 0.50 (p=0.02) based upon only 22 subjects with detectable dioxin levels. Based on the anticipated sample size (67 per group) and analogous stratification, there will be sufficient power to detect meaningful correlations.

All analyses will be performed with and without subjects whose dioxin levels fall below the minimum detectable value. For those who fall below the limit, the minimum detectable value divided by the square root of 2 will be imputed. For each measure, the Mantel-Haenszel chi-square statistic will be used to determine whether the proportion falling below the limit differs systematically across the three exposure levels.

TABLE 5. LIST OF RESIDENCES FOR 20 VIETNAMESE

I.D	X Y coordinate	Years of residence
0001	: XS 070 332	1945 - 1993
0002	: CP 036 539	1971 - 1972
	: BN 815 785	1972 - 1974
	: AN 856 096	1974 - 1993
0003	: WS 602 150	1930 - 1993
0004	: WS 568 728	1960 - 1993
0005	: XR 082 305	1957 - 1993
0006	: XS 587 578	1938 - 1993
0007	: WR 130 975	1954 - 1975
	WS 300 161	1975 - 1979
	WR 130 975	1979 - 1993
0009	: XS 550 472	1948 - 1993
0010	: YS 124 999	1963 - 1993
0011	: AN 856 096	1951 - 1975
	: ZS 039 794	1975 - 1977
	: BP 487 089	1977 - 1982
	: AN 856 096	1982 - 1993
0012	: WQ 282 755	1964 - 1993
0013	: YS 124 999	1975 - 1978
0014	: XS 135 422	1951 - 1968
	VS 893 162	1968 - 1993
0015	: YT 086 118	1956 - 1993
0016	: XS 511 318	1963 - 1993
0017	: WS 443 658	1943 - 1993
0018	: WR 563 852	1929 - 1993
0019	: XS 231 338	1941 - 1993
0020	: XS 030 911	1930 - 1993

Table 6. Example of Exposure Assessment for Twenty Subjects in IARC Case-Control Study

Rec.	ID	Grid	X	Y	Yearin	Yearout	Exposure	Hits5km	Hits10km	Hits15km
1	1	XS	7	33.2	65	93	0.7073	9	11	51
2	2	CP	3.6	53.9	71	72	0.0025	0	0	0
3	2	BN	81.5	78.5	72	74	0	0	0	0
4	2	AN	85.6	9.6	74	93	0	0	0	0
5	3	WS	60.2	15	65	93	1.7966	1	34	157
6	4	WS	56.8	72.8	65	93	0.2709	4	4	9
7	5	XR	8.2	30.5	65	93	0.255	1	6	21
8	6	XS	58.7	57.8	65	93	0.4699	0	11	45
9	7	WR	13	97.5	65	75	0.0726	0	1	7
10	7	WS	30	16.1	75	79	0	0	0	0
11	7	WR	13	97.5	79	93	0	0	0	0
12	9	XS	55	47.2	65	93	2.2853	26	52	75
13	10	YS	12.4	99.9	65	93	4.0243	33	136	251
14	11	AN	85.6	9.6	65	75	0.198	0	4	17
15	11	ZS	3.9	79.4	75	77	0	0	0	0
16	11	BP	48.7	8.9	77	82	0	0	0	0
17	11	AN	85.6	9.6	82	93	0	0	0	0
18	12	WQ	28.2	75.5	65	93	0.4111	1	19	33
19	13	YS	12.4	99.9	75	78	0	0	0	0
20	14	XS	13.5	42.2	65	68	0.0336	0	0	4
21	14	VS	89.3	16.2	68	93	1.242	19	40	43
22	15	YT	8.6	11.8	65	93	7.2467	91	174	273
23	16	XS	51.1	31.8	65	93	0.831	6	21	60
24	17	WS	44.3	65.8	65	93	0.0327	0	1	3
25	18	WR	56.3	85.2	65	93	0.0998	0	0	11
26	19	XS	23.1	33.8	65	93	0.3965	1	10	34
27	20	XS	3	91.1	65	93	0.5766	6	9	27

PROJECT 6: Planning and development of epidemiological research priorities

Background and Objectives

Throughout this proposal we have described a rich and voluminous collection of data resources from which it is possible, in principle, to reconstruct the herbicide exposure opportunities of hundreds of thousands of veterans. We have proposed a series of research projects that address our principal scientific concern about the accuracy of such assignments and the direction and degree of misclassification which must be adequately resolved before quantitative exposure assessment methods can be routinely applied in epidemiologic studies (Project 1). We have also proposed several laboratory-based methodological studies both for providing further insight into the sensitivity of the records-based exposure opportunity index and for quantifying the extent to which such assays remain relevant in the planning and setting of priorities for future research (Projects 4 & 5). We have proposed a project (Project 2) which will explore the dynamics of the interview method amongst Vietnam veterans and will provide a set of validated measures of important independent variables, intervening variables and confounders which can be used in epidemiological studies. Finally, we have proposed to carry out an extensive analysis of the availability and the quality of data held by the National Personnel Records Center, developed a user-friendly guide to these records and suitable abstraction forms (Project 3). This information should help overcome a now rather formidable obstacle to the successful carrying out of research on veterans.

The primary objective of Project 6 is to synthesize the findings of Projects 1-5 and the state-of-the-art of knowledge that is available in month 30 of this proposed grant. We plan to have intensive interaction, including an intensive structured 5-day workshop, with the collaborators on this project, with its Advisory Board, with the NAS Advisory Committee and with three scientists invited to join the project in the third year in a formal consultative arrangement, in order to arrive at a proposed set of priorities and designs for future epidemiological studies.

Methods

The development of research priorities is a complex adventure encompassing a careful balance of pragmatic realities, wished-for opportunities and methodological constraints. We will have established an Advisory Board, chaired by Admiral Elmo Zumwalt, that has deep insight into the problems that exist, the questions that need to be answered, the history of the problem and the realities of what can be accomplished. John F. Sommer, Executive Director of the Washington Office of The American Legion has also agreed to serve. We have complemented this Advisory Board with a three-person Scientific Advisory Board (Boffetta, Garfinkle and Zahm) that has extensive experience in the design and execution of epidemiological research. The principal collaborators on this project bring to it a wide-range of relevant experiences and expertise, as does the NAS Advisory Committee itself.

We plan to develop materials that will present a synthesis of all research findings to these parties in a user-friendly format. "Unsynthesized" results will, of course, also be available upon request. We intend to develop a compilation of those databases on health

outcomes which are already available and could be the basis of future research as well. The major tasks for which the three consulting scientists will be contracted is preparation for this workshop. We are particularly interested in having state-of-the-art data on health effects of herbicides in general, on other relevant clinical data and on methodological issues. We will ask the consultants each to carve out an area of "interest" in which to complete an intensive analytical review, to study our findings carefully and to develop potential hypotheses that could reasonably be the basis of future epidemiological work. We have not yet selected potential candidates and we have not ruled out the possibility that a Science Panel member or statistical consultant could fill one of these positions.

The Principal Investigator, who has extensive expertise in knowing how to efficiently organize experts and have them work together to produce scientifically valid results (e.g., the Encyclopaedia of Occupational Health and Safety, 4th edition, which she edited is a peer-reviewed four volume, 102 chapter work with more than 1500 contributors), will carry out a semi-structured interview with each Advisor and collaborator in order to determine the key issues and the key questions that exist. Each person will be asked to provide ideas and suggestions that she or he may already have about possible priorities and studies. J. Stellman will synthesize the results of these telephone interviews and distribute the results of these interviews to all parties. A proposed agenda for the workshop, in consultation with the three Advisory Board members, will be developed, and written responses to it will be solicited. The responses will be reviewed and suggestions incorporated. A conference call will then be held in order discuss the agenda and receive suggestions for change. These suggestions will then be reviewed by the Advisory Board and the Scientific Advisory Board and a final agenda agreed upon.

The 5-day workshop will be held either at Columbia University's Arden House executive retreat facilities or at the National Academy of Sciences. The decision for location will be arrived at in consultation with the NAS. The goal of the workshop will be to develop a specific priority list for research and suggested epidemiological designs. A sufficient number of computers and printers will be available, as well as other conference aids, so that written products can be expected to result from this effort. Each of the people participating in this workshop has had experience in similar workshops and advisory group meetings and sufficient pre-planning will have taken place to assure orderly and productive procedings.

This various parties should be well-acquainted by month 30. They will have attended two yearly meetings and two meetings with the NAS committee will have taken place. The collaborators on the grant will have, of course, had multiple opportunities for interaction and exchange of ideas. Thus, the group should be in an excellent position to "get to work" at the workshop.

It is anticipated that the workshop will spend the first 1.5 days deliberating on overall priorities and possibilities for future research and will then break up into smaller working groups that will focus on "filling in" details for proposed research. It may be that the resources of the University might be needed or that contact with other colleagues may be desired and adequate communications systems will be available. The last 1.5 days will be spent in a group critique of the work of the subgroups, revision by the subgroups and the submission of "final" subgroup reports. These will be synthesized by Stellman and Stellman and circulated to all participants for final comment.

The results of the workshop will then be summarized in a final report, which will also summarize the results of each of the proposed project and will be presented to the NAS for its feedback. During the course of the work, it is anticipated that results of various projects will be submitted in the form of journal articles to the NAS for approval for publication.