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The Biomarkers of Exposure and Effect in Agriculture (BEEA) Study: Rationale, design, methods, and participant characteristics

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Abstract

Agricultural exposures including pesticides, endotoxin, and allergens have been associated with risk of various cancers and other chronic diseases, although the biological mechanisms underlying these associations are generally unclear. To facilitate future molecular epidemiologic investigations, in 2010 the study of Biomarkers of Exposure and Effect in Agriculture (BEEA) was initiated within the Agricultural Health Study, a large prospective cohort in Iowa and North Carolina. Here the design and methodology of BEEA are described and preliminary frequencies for participant characteristics and current agricultural exposures reported. At least 1,600 male farmers over 50 years of age will be enrolled in the BEEA study. During a home visit, participants are asked to complete a detailed interview about recent agricultural exposures and provide samples of blood, urine, and (since 2013) house dust. As of mid-September 2014, a total of 1,233 participants have enrolled. Most of these participants (83%) were still farming at the time of interview. Among those still farming, the most commonly reported crops were corn (81%) and soybeans (74%) and the most frequently noted animals were beef cattle (35%) and hogs (13%). There were 861 (70%) participants who reported occupational pesticide use in the 12 months prior to interview; among these participants, the most frequently noted herbicides were glyphosate (83%) and 2,4-D (72%), and most commonly reported insecticides were malathion (21%), cyfluthrin (13%), and permethrin (12%). Molecular epidemiologic investigations within BEEA have the potential to yield important new insights into the biological mechanisms through which these or other agricultural exposures influence disease risk.

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Keywords

Pesticides; agriculture; biomarkers; molecular epidemiology

Introduction

Numerous studies among farmers, including the Agricultural Health Study (AHS), have demonstrated an excess of some cancers including lymphoid neoplasms and prostate cancer and a lower burden of others such as lung cancer relative to the general population (Blair and Beane Freeman, 2009; Koutros et al, 2010; Alavanja and Bonner, 2012). Farmers perform a wide range of tasks while raising crops and/or animals that may involve exposure to chemical and biological agents. Epidemiologic investigations are conducted to identify occupational and environmental exposures amongst individuals residing or working on farms that might influence risk of cancer development (Blair and Beane Freeman, 2009; Alavanja and Bonner, 2012) or other health endpoints such as respiratory diseases (ATS, 1998; May et al, 2012) and neurodevelopmental effects (London et al, 2012; Burns et al, 2013). Specific exposures that have been assessed include pesticides, endotoxin and other bioaerosols, zoonotic viruses and other bloodborne pathogens, solvents, welding fumes, and diesel exhaust (Blair and Beane Freeman, 2009). Some farm exposures may be associated with an increased risk of certain malignancies such as lindane and non-Hodgkin lymphoma (Schinasi and Leon, 2014; Alavanja et al, 2014), whereas other exposures may be associated with reduced risk such as endotoxin and lung cancer (Lenters et al, 2010; Beane Freeman et al, 2012).

Although there has been substantial progress in characterizing agricultural exposures that are associated with cancer risk, our ability to infer causality is often limited by lack of a clear understanding of potential biological mechanisms underlying the association between a given exposure and outcome. Molecular epidemiologic investigations have the potential to provide important new insights into the biological plausibility of observed associations and to characterize possible pro- or anti-carcinogenic mechanisms of action. To facilitate such research in the future, a cross-sectional molecular epidemiology study was initiated with an anticipated enrollment of at least 1,600 male farmers from the AHS, a large prospective cohort in Iowa and North Carolina. This article describes the methodology for this effort, the study of Biomarkers of Exposure and Effect in Agriculture (BEEA), with a particular focus on procedures for subject enrollment, data and specimen collection, and specimen processing and storage. In addition, the basic characteristics of the BEEA study participants enrolled to date are summarized, and frequencies for some current exposures reported.

Methods

Study population and recruitment for BEEA

Male private applicators (nearly all farmers) are being recruited for the BEEA study from among participants in the AHS. The design of the AHS cohort was previously described in detail (Alavanja et al, 1996). Briefly, individuals were recruited and completed an enrollment questionnaire between December 1993 and December 1997 while attending

certification sessions for licenses to apply restricted use pesticides. The AHS enrollment (Phase 1) questionnaires solicited information regarding use of specific pesticides, crops grown and livestock raised, other activities on the farm such as repairing equipment, welding, and painting, use of personal protective equipment, personal history of medical conditions, family history of cancer, health-related behaviors including smoking and alcohol consumption, height and weight, and demographic characteristics. Additional follow-up questionnaires were administered in Phase 2 (1998–2003) and Phase 3 (2005–2010) that collected information regarding exposures and health conditions since enrollment. These questionnaires are available online (<http://aghealth.nih.gov>).

Male farmers in the AHS are eligible to participate in BEEA if they (1) are over 50 years of age; (2) have never been diagnosed with cancer (other than non-melanoma skin cancer); (3) completed the questionnaires administered during Phases 1–3 of the study; (4) still reside in Iowa or North Carolina; and (5) do not have a blood clotting disorder such as hemophilia. On an approximately annual basis, potentially eligible participants are identified and selected for the recruitment list based on current age, cancer history as of the most recent linkages with the Iowa and North Carolina state cancer registries, and vital status as ascertained from the state mortality registries and the National Death Index. Recruitment for BEEA is ongoing throughout the calendar year. Potentially eligible participants are contacted by mail and by phone to verify eligibility, assess willingness to participate, and schedule a phlebotomist visit to the participant's home. Prior to the home visit, the participant receives another mailing with the consent form, instructions about preparing for the interview, and a kit for collection of a first morning void urine sample on the day of the home visit. Enrollment of participants in BEEA began in June 2010. Although enrollment proceeded more rapidly in Iowa than North Carolina, plans for future recruitment are to enroll a higher proportion of participants from North Carolina up to approximately one-third of the total enrollment in BEEA to reflect the overall distribution of the AHS cohort.

Interview and specimen collection, processing, and storage

At the beginning of the visit to the participant's home, the phlebotomist reviews the consent form with the participant and obtains written informed consent. After again verifying eligibility, the phlebotomist conducts a computer-assisted personal interview (CAPI) and collects the non-fasting blood sample, the first morning void urine sample, and (since November 2013) a vacuum dust sample from the participant's home. The CAPI solicits information regarding recent exposures and health conditions, such as (1) specific pesticides used in the last 12 months, including the number of days of use and dates of the most recent applications; (2) use of personal protective equipment while handling pesticides; (3) crops grown and animals raised, including time spent working in animal confinement facilities and performing specific tasks related to crop and animal production; (4) other farm exposures such as welding, painting, and repairing engines; (5) non-farming jobs; (6) history of medical conditions including recent infections; (7) current medications; (8) self-reported current height and weight; and (9) alcohol consumption and tobacco use. The frequencies reported in this article are based on the information from this interview, with the exception of race and level of education which were ascertained from the AHS enrollment questionnaire. During the home visit, the phlebotomist also records the geographic

coordinates of the participant's residence using a Garmin® 76CSx handheld global positioning system (GPS) device. The phlebotomist is trained in all study procedures including interviewing techniques, collecting and transporting biospecimens and vacuum dust, and recording GPS coordinates. With the participants' permission, the interviews are recorded and some are randomly selected for quality control review.

An overview of the specimens collected from participants in BEEA is shown in Table 1. Participants are asked to provide up to 60 ml of blood in multiple tubes for isolation of serum, heparin- and ethylenediaminetetraacetic acid (EDTA)-treated plasma, buffy coat, and red blood cells. Acid citrate dextrose (ACD) tubes are collected for cryopreservation of whole blood aliquots, and PAXgene tubes are collected to enable isolation and purification of intracellular RNA and microRNA. Participants are asked to store the urine sample in a refrigerator until it is given to the phlebotomist during the home visit. Dust samples are collected from the vacuum cleaner that is used most frequently in the participant's home using a standard protocol. For vacuum cleaners with disposable bags, the entire bag is collected, wrapped in aluminum foil, and stored in a sealed plastic bag. For canister vacuum cleaners, the contents of the canister are deposited onto aluminum foil, wrapped, and stored in a sealed plastic bag. When participants are contacted prior to the home visit, they are asked not to discard the vacuum dust before the scheduled visit so that it can be collected by the phlebotomist at that time.

After completing the home visit, the phlebotomist transports the blood and urine samples in a temperature-controlled environment to a local shipping center, where they are sent via overnight delivery to a central processing facility. These samples are kept cold during transport and shipment, with the exception of the ACD tubes, which are shipped at ambient temperature. Upon receipt at the processing lab, samples are aliquoted according to a standard protocol. Blood from the ACD tubes is treated with dimethyl sulfoxide (DMSO), aliquoted, and cryopreserved using a controlled rate freezer to -90°C and then stored in a liquid nitrogen freezer. The PAXgene tubes are frozen at -20°C for 24 hr and then transferred to a -80°C freezer. All other samples are frozen at -80°C after aliquoting. The dust samples are shipped at ambient temperature approximately monthly to a separate processing facility, where they are sieved ($<150\text{ }\mu\text{m}$), aliquoted, and frozen at -80°C for long-term storage.

Human subjects' protection

All subjects provided written informed consent before participating in BEEA. All study procedures were approved by the NCI Special Studies Institutional Review Board and the Institutional Review Boards of other relevant organizations.

Results

As of September 15, 2014 (the latest date for which cleaned data were available from the BEEA interview), a total of 1,233 participants were enrolled in BEEA. Among those who could be contacted and who were confirmed as eligible at that time, 56% agreed to participate in BEEA. Selected demographic and other characteristics of BEEA participants enrolled as of this date are reported in Table 2. The median age of participants was 64 years

(interquartile range 58–72 years), and most (83%) were still farming at the time of enrollment (86% in Iowa and 69% in North Carolina). Based on self-reported information from the phlebotomist's interview, compared to participants in Iowa, those in North Carolina tended to be older, had lower BMI, and had lower alcohol consumption. Use of tobacco products was more common in North Carolina than Iowa. Participants in BEEA were similar to male farmers in the overall AHS cohort with respect to demographic characteristics including age (mean of 64 years overall in the AHS), race (95% white), and level of education (41% with some education beyond a high school degree).

Frequencies for the most common crops grown and animals raised in the 12 months prior to the phlebotomist's interview are shown in Table 3. The most common crops grown by BEEA participants who were still farming were corn and soybeans (81 and 74%, respectively). The proportion of current farmers growing corn, soybeans, alfalfa/hay, and oats was higher in Iowa than North Carolina, whereas a greater proportion of current farmers in North Carolina were growing wheat. The most common animals raised for income were beef cattle and hogs (35 and 13%, respectively). Among the participants raising animals for income, most reported having a large number of animals on the farm. The median (interquartile range) number of animals raised was 60 (27–150) for beef cattle farmers and 3,000 (600–8,000) for hog farmers. The proportion of current farmers raising hogs was higher in Iowa than North Carolina (15 and 6%, respectively), whereas raising poultry was more common in North Carolina than Iowa (12 and 5%, respectively).

Overall, 70% of participants in BEEA reported occupational pesticide use in the 12 months prior to interview (74% in Iowa and 51% in North Carolina). Table 4 shows the frequencies for herbicides, insecticides, and fungicides reported most commonly (5% overall) among current applicators. Glyphosate and 2,4-D were the most frequently noted herbicides (83 and 72%, respectively). The herbicide picloram was used by 35% of these participants; it was generally used in combination with 2,4-D. Use of atrazine was also relatively common (32%). For insecticides, malathion [an organophosphate OP] was used most frequently (21%), followed by the synthetic pyrethroids cyfluthrin (13%) and permethrin (12%). Pyraclostrobin was the most commonly reported fungicide (5%). Most of these pesticides were used by a higher proportion of current applicators in Iowa than in North Carolina, with the exception of chlorpyrifos, an OP insecticide (11 and 5% in North Carolina and Iowa, respectively).

Discussion

Molecular epidemiologic investigations can complement classical epidemiologic studies of the relations between agricultural exposures and cancer or other health outcomes by informing our understanding of the biological mechanisms through which these exposures may influence risk. The BEEA study has several notable strengths as a resource for future investigations of pro- and anti-carcinogenic effects of agricultural exposures. Because all of the participants in BEEA completed each of the questionnaires administered previously in Phases 1–3 of the AHS and at the time of phlebotomy, occupational exposure to specific pesticides, other agricultural exposures, and health and lifestyle characteristics can be assessed comprehensively over their working lifetime. This adds a historical exposure

assessment component to the cross-sectional design (Dosemeci et al, 2002; Coble et al, 2011). Further, various types of specimens are being collected that are suitable for a wide range of intermediate biomarkers that may potentially help to explain observed associations between agricultural exposures and cancer or other chronic diseases, including measurements performed using newly emerging technologies such as metabolomics platforms and gene expression arrays. Finally, with an anticipated enrollment of at least 1,600 subjects, the study will be sufficiently large for targeted investigations of subgroups of participants with specific agricultural exposures.

Given the wide range of tasks associated with growing crops and raising animals, farmers may be exposed to a variety of agents that may influence risk of various malignancies. Pesticides are an important class of agricultural exposures that includes herbicides, insecticides, fungicides, and fumigants, many of which are known to result in adverse toxic effects to humans following high-dose acute and chronic exposures (Blair et al, 2015; Thundiyil et al, 2008). Pesticides have diverse chemical structures and exhibit various biological modes of actions in both target and non-target organisms (Casida, 2012; Wolansky and Tornero-Velez, 2013). A variety of mechanisms besides overt genotoxicity may be involved in pesticide-mediated carcinogenesis, including oxidative stress, inflammatory response and immune perturbations, endocrine disruption, and aberrant epigenetic mechanisms (Alavanja and Bonner, 2012; Alavanja et al, 2013).

One early area of investigation in BEEA will focus on agricultural exposures that may be associated with risk of multiple myeloma and its precursor condition, monoclonal gammopathy of undetermined significance (MGUS). An elevated risk of multiple myeloma was observed fairly consistently among farmers over the past 30 years (Perrotta et al, 2008). Some specific agricultural exposures have been linked to multiple myeloma risk (Merhi et al, 2007); in the AHS, farmers who used permethrin had an increased risk of multiple myeloma compared with those who did not (Rusiecki et al, 2009; Alavanja et al, 2014). However, overall the evidence is still inconclusive. In a prior study in the AHS (Landgren et al, 2009), the age-adjusted prevalence of MGUS was noted to be 1.9 fold higher (95% CI, 1.3-fold to 2.7-fold) among 555 male pesticide applicators compared to a demographically similar group of men from Olmsted County, Minnesota. To follow up on this observation and investigate specific agricultural exposures that may be associated with MGUS, an investigation of this myeloma precursor is planned within the larger BEEA study population.

Future investigations in BEEA will also focus on leukocyte telomere length as an intermediate biomarker linking pesticide exposures and cancer risk. Telomeres are repetitive nucleotide sequences at chromosome ends that are essential for maintaining genomic integrity; telomere attrition occurs gradually over time due to inefficient replication at the linear ends of DNA with each cell division. Previous epidemiologic studies have linked altered leukocyte telomere length with risk of various malignancies (reviewed in Ma et al, 2011; Wentzensen et al, 2011), and recent investigations in the AHS have demonstrated that exposure to the herbicide 2,4-D and possibly some other pesticides is associated with shorter telomere length in buccal epithelial cells and peripheral blood leukocytes (Hou et al, 2013; Andreotti et al, 2015). Notably, a recent assessment of the carcinogenicity of 2,4-D conducted by the International Agency for Research on Cancer found that there was

mechanistic evidence that 2,4-D can induce oxidative stress in humans (Loomis et al, 2015), and oxidative stress may in turn influence telomere length (von Zglinicki, 2002). As such, confirmation of these findings and investigation of telomere length in relation to other agricultural exposures in BEEA is warranted.

Farmers can also be highly exposed to organic dusts and allergens while working with certain crops and animals in various agricultural operations (ATS, 1998; May et al, 2012; Hawley et al, 2015), and there is some evidence to suggest that these exposures may influence the risk of certain cancers. In a previous investigation in the AHS (Beane Freeman et al, 2012), the risk of lung cancer was reduced among farmers raising poultry and larger numbers of livestock, possibly as a result of increased exposure to endotoxin. More recently, Hofmann et al (2015) found a reduced risk of lymphoid malignancies among AHS participants who reported having allergy symptoms at baseline, in particular among farmers handling stored grains or hay. Within BEEA, the potential biological mechanisms through which endotoxin and other bioaerosol exposures and allergy-related manifestations influence cancer risk in a farming population will be examined.

In summary, conducting molecular epidemiologic studies provides insights into the biologic mechanisms underlying associations between agricultural exposures and various cancers or other chronic diseases. Although conducted within a farming population, the results may have implications for larger populations. For example, in the National Health and Nutrition Examination Survey, the majority of the U.S. population with no occupational exposure to pesticides displayed detectable levels of various pesticide metabolites in their urine (Barr et al, 2010; 2011). The BEEA study will be an important resource for future work in this area.

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Table 1

Summary of samples collected from BEEA participants

Collection	Stored specimens	N (%)
Blood tubes		
Red top	Serum, clot	1,222 (99.1)
Green top (heparin)	Heparin plasma, buffy coat, RBCs	1,220 (98.9)
Lavender top (EDTA)	EDTA plasma, buffy coat, RBCs	1,218 (98.8)
Yellow top (ACD)	DMSO-treated whole blood	1,201 (97.4)
PAXgene	Whole blood	1,217 (98.7)
Urine	Aliquots	1,220 (98.9)
Vacuum dust	Aliquots	189 (94.0) ^a

Notes: EDTA, ethylenediaminetetraacetic acid; ACD, acid citrate dextrose; DMSO, dimethyl sulfoxide

^a Collection began in November 2013; % is based on 201 attempted dust collections.

Table 2Selected characteristics of AHS participants enrolled in BEEA^a

Characteristic	Overall N (%)	Iowa N (%)	North Carolina N (%)
No. of participants	1,233	1,005	228
Age at phlebotomy, median (interquartile range)	64 (58–72)	63 (57–72)	67 (60–74)
Race			
White	1,201 (97.4)	992 (98.7)	209 (91.7)
Other/missing	32 (2.6)	13 (1.3)	19 (8.3)
Currently farming			
Yes	1,021 (82.8)	864 (86.0)	157 (68.9)
No	212 (17.2)	141 (14.0)	71 (31.1)
Education			
Less than high school	56 (4.5)	39 (3.9)	17 (7.5)
High school graduate	565 (45.8)	478 (47.6)	87 (38.2)
Some college or vocational school	295 (23.9)	246 (24.5)	49 (21.5)
College graduate	282 (22.9)	220 (21.9)	62 (27.2)
Other/missing	35 (2.8)	22 (2.2)	13 (5.7)
Body mass index			
<25 kg/m ²	191 (15.5)	142 (14.1)	49 (21.5)
25–29.9 kg/m ²	550 (44.6)	451 (44.9)	99 (43.4)
30–34.9 kg/m ²	343 (27.8)	293 (29.2)	50 (21.9)
35 kg/m ²	149 (12.1)	119 (11.8)	30 (13.2)
Current use of any tobacco products			
No	1,095 (88.8)	911 (90.7)	184 (80.7)
Yes	138 (11.2)	94 (9.4)	44 (19.3)
Servings of alcoholic beverages in the past 7 days ^b			
0	591 (47.9)	431 (42.9)	160 (70.2)
1–2	236 (19.1)	214 (21.3)	22 (9.7)
3–5	161 (13.1)	147 (14.6)	14 (6.1)
6–9	108 (8.8)	94 (9.4)	14 (6.1)
10–19	87 (7.1)	77 (7.7)	10 (4.4)
20	50 (4.1)	42 (4.2)	8 (3.5)

^aDetermined based on the information collected during the interview at the time of the home visit, with the exception of race and level of education which were ascertained from the enrollment questionnaire. The results are reported as frequencies (%) unless otherwise noted.

^bOne serving of an alcoholic beverage was defined as 12 fluid ounces of beer, 5 fluid ounces of wine, or 1.5 fluid ounces of hard liquor.

Table 3Current farm characteristics of BEEA participants who are still farming (N=1,021)^a

Farm characteristics	Overall N (%)	Iowa N (%)^a	North Carolina N (%)^b
<i>Crops</i>			
Corn	823 (80.6)	764 (88.4)	59 (37.6)
Soybeans	759 (74.3)	682 (78.9)	77 (49.0)
Alfalfa or hay	448 (43.9)	398 (46.1)	50 (31.9)
Oats	123 (12.0)	114 (13.2)	9 (5.7)
Wheat	64 (6.3)	10 (1.2)	54 (34.4)
<i>Livestock or animals</i>			
Beef cattle	359 (35.2)	308 (35.7)	51 (32.5)
Hogs	135 (13.2)	125 (14.5)	10 (6.4)
Sheep or Goats	59 (5.8)	52 (6.0)	7 (4.5)
Dairy Cattle	57 (5.6)	51 (5.9)	6 (3.8)
Poultry	57 (5.6)	39 (4.5)	18 (11.5)

^aDetermined based on the information collected during the interview at the time of the home visit.^bState-specific % were based on the total number of participants still farming in Iowa (N=864) and in North Carolina (N=157).

Table 4

Reported herbicides, insecticides, and fungicides among BEEA participants with occupational pesticide use in the last 12 months (N=861)^a

Chemicals by class	Overall N (%)	Iowa N (%) ^a	North Carolina N (%) ^b
<i>Herbicides</i>			
Glyphosate	717 (83.3)	625 (83.9)	92 (79.3)
2,4-D	616 (71.5)	567 (76.1)	49 (42.2)
Picloram	303 (35.2)	299 (40.1)	4 (3.5)
Atrazine	276 (32.1)	253 (34.0)	23 (19.8)
Triclopyr	110 (12.8)	99 (13.3)	11 (9.5)
Acetochlor	98 (11.4)	97 (13.0)	1 (0.9)
Mesotrione	91 (10.6)	88 (11.8)	3 (2.6)
Metolachlor	86 (10.0)	74 (9.9)	12 (10.3)
Dicamba	83 (9.6)	76 (10.2)	7 (6.0)
Isoxaflutole	70 (8.1)	70 (9.4)	0 (0.0)
Clethodim	70 (8.1)	70 (9.4)	0 (0.0)
S-dimethenamid	59 (6.9)	58 (7.8)	1 (0.9)
Thiencarbazone-methyl	52 (6.0)	52 (7.0)	0 (0.0)
Acifluorfen	43 (5.0)	42 (5.6)	1 (0.9)
Clopyralid	43 (5.0)	42 (5.6)	1 (0.9)
<i>Insecticides</i>			
Malathion	183 (21.3)	174 (23.4)	9 (7.8)
Cyfluthrin	110 (12.8)	107 (14.4)	3 (2.6)
Permethrin	100 (11.6)	91 (12.2)	9 (7.8)
Tebupirimfos	60 (7.0)	60 (8.1)	0 (0.0)
Pyrethrins	48 (5.6)	48 (6.4)	0 (0.0)
Chlorpyrifos	47 (5.5)	34 (4.6)	13 (11.2)
Clothianidin	45 (5.2)	45 (6.0)	0 (0.0)
<i>Fungicides</i>			
Pyraclostrobin	45 (5.2)	43 (5.8)	2 (1.7)

^a Determined based on the information collected during the interview at the time of the home visit.

^b State-specific % were based on the total number of participants who reported occupational pesticide use in the last 12 months in Iowa (N=745) and in North Carolina (N=116).