Original Investigation

Elevated Serum Pesticide Levels and Risk for Alzheimer Disease

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IMPORTANCE The causes of late-onset Alzheimer disease (AD) are not yet understood but likely include a combination of genetic, environmental, and lifestyle factors. Limited epidemiological studies suggest that occupational pesticide exposures are associated with AD. Previously, we reported that serum levels of dichlorodiphenyldichloroethylene (DDE), the metabolite of the pesticide dichlorodiphenyltrichloroethane (DDT), were elevated in a small number of patients with AD (n=20).

OBJECTIVE To evaluate the association between serum levels of DDE and AD and whether the apolipoprotein E (*APOE*) genotype modifies the association.

DESIGN, SETTING, AND PARTICIPANTS A case-control study consisting of existing samples from patients with AD and control participants from the Emory University Alzheimer's Disease Research Center and the University of Texas Southwestern Medical School's Alzheimer's Disease Center. Serum levels of DDE were measured in 79 control and 86 AD cases.

MAIN OUTCOMES AND MEASURES Serum DDE levels, AD diagnosis, severity of AD measured by the Mini-Mental State Examination score, and interaction with *APOE4* status.

RESULTS Levels of DDE were 3.8-fold higher in the serum of those with AD (mean [SEM], 2.64 [0.35] ng/mg cholesterol) when compared with control participants (mean [SEM], 0.69 [0.1] ng/mg cholesterol; $P < .001$). The highest tertile of DDE levels was associated with an odds ratio of 4.18 for increased risk for AD (95% CI, 2.54-5.82; $P < .001$) and lower Mini-Mental State Examination scores (−1.605; range, −3.095 to −0.114; $P < .0001$). The Mini-Mental State Examination scores in the highest tertile of DDE were −1.753 points lower in the subpopulation carrying an *APOE* ε4 allele compared with those carrying an *APOE* ε3 allele (P interaction = .04). Serum levels of DDE were highly correlated with brain levels of DDE ($\rho = 0.95$). Exposure of human neuroblastoma cells to DDT or DDE increased levels of amyloid precursor protein.

CONCLUSIONS AND RELEVANCE Elevated serum DDE levels are associated with an increased risk for AD and carriers of an *APOE4* ε4 allele may be more susceptible to the effects of DDE. Both DDT and DDE increase amyloid precursor protein levels, providing mechanistic plausibility for the association of DDE exposure with AD. Identifying people who have elevated levels of DDE and carry an *APOE* ε4 allele may lead to early identification of some cases of AD.

Editorial

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Alzheimer disease (AD) is the most common neurodegenerative disease worldwide and cases are expected to increase 3-fold over the next 40 years.[1] The most common form of AD is the late-onset form, which typically develops after 60 years of age. The etiological factors of late-onset AD are not yet completely understood but include genetic, environmental, and lifestyle factors that influence a person's risk for developing the disease.[2] Although there is a growing list of AD susceptibility genes, only having an apolipoprotein E4 (*APOE4*) allele has a relatively strong effect (relative risk approximately 2-3), and, cumulatively, the more than 10 genes identified thus far account for only less than half of AD cases.[3] To our knowledge, few studies have explored the potential of environmental exposures to contribute to AD, but occupational exposure to metals, solvents, and pesticide is reported to be a potential environmental contributor.[4,5] Previously, we reported that serum levels of p,p'-dichlorodiphenyldichloroethylene (DDE), a metabolite of the organochlorine pesticide dichlorodiphenyltrichloroethane (DDT), were significantly higher in a small cohort (n = 20) of patients with AD compared with control participants, and that there was a significant association between DDE levels and a diagnosis of AD.[6] In the present study, we evaluated the associations between serum DDE levels, AD, and Mini-Mental State Examination (MMSE) scores in a larger number of cases and control participants from 2 geographical sites, and we explored differential susceptibility by *APOE4* genotype status. We also examined the relationship between brain and serum levels of DDE and whether DDT or DDE alters the expression of the amyloid precursor protein (APP) in cultured neuronal cells.

Methods

Study Population

Existing serum samples were obtained from control participants and patients with AD who were evaluated in the Alzheimer's Disease Research Centers at the University of Texas Southwestern Medical Center (UTSW) and Emory University between 2002 and 2008. Participants who provided samples were diagnosed and assigned to AD or normal control groups based on consensus diagnosis. Normal control participants were determined to have normal neurological/clinical examinations and neuropsychological functioning findings on standardized testing. The inclusion criteria were as follows: (1) MMSE score of 28 to 30 for the control participants, (2) no structural brain abnormalities indicated by magnetic resonance imaging; and/or (3) normal general neurological examination; and (4) normal Consortium to Establish a Registry for Alzheimer's Disease battery results. Patients with AD were diagnosed by applying National Institute of Neurological and Communicative Disorders-Alzheimer's Disease and Related Disorders Association criteria for probable AD based on neurological and neuropsychological examination results, brain imaging, and laboratory assessments to rule out other causes of dementia at both UTSW and Emory University. Blood samples for serum preparation and genotyping were generally taken at enrollment along with MMSE. All participants had *APOE* genotype determined by standard TaqMan polymerase chain reaction.

Data from 43 control samples and 20 AD samples from UTSW reported in our first study were included in this analysis.[6] An additional 11 control and 41 AD samples were provided by UTSW, and 25 control and 25 AD samples were provided by Emory University. Serum samples were randomly selected from existing samples collected between 2002 and 2008, and an attempt was made to match these based on age, sex, and race/ethnicity.

Matched brain and serum samples of patients with AD were obtained from the Alzheimer's Disease Center at Washington University to determine whether serum levels of DDE correlated with brain levels. All samples (n = 11; average age = 85.7 years) were from patients diagnosed as having AD by National Institute of Neurological and Communicative Disorders-Alzheimer's Disease and Related Disorders Association criteria and verified histopathologically following death. Blood samples were taken an average of 193 days before death and brain samples were obtained after an average postmortem interval of 12 hours.

The institutional review boards of UTSW, Washington University, Emory University, and the Robert Wood Johnson Medical School approved all of the protocols and procedures. All participants reviewed and signed written approved informed consent documents.

Assessment of Pesticide Levels

Serum DDE levels were determined by gas chromatography/mass spectrometry, as described previously,[6,7] and expressed in terms of free cholesterol levels. The limit of detection for DDE was approximately 100 pg/mL. For brain pesticide determination, approximately 150 mg of temporal cortex was sonicated in a 1:1 mixture of acetone and hexane containing 5 µL of an internal standard (4-4'-DDT ^{13}C, 1 mg/mL). Following an overnight incubation, the sample was centrifuged for 10 minutes at 3500 rpm and supernatant removed. The extraction procedure was repeated 4 times and the extract reduced by evaporation under nitrogen. The dried residue was reconstituted in acetone:hexane and applied to a solid-phase extraction column containing 5 g of Florisil and 1.5 g of anhydrous sodium sulfate preconditioned with 8 mL of hexane. Analytes were eluted with methyl tert-butyl ether, evaporated to dryness, and reconstituted in 1.8 mL of hexane for GC/MS analysis, as described previously.[6,7]

In Vitro Exposure to DDT/DDE and Analysis of APP Levels

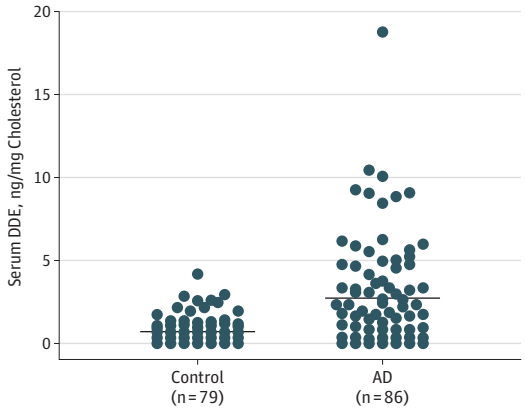
SH-SY5Y cells (ATCC) were differentiated by reducing serum concentration to 1% and the addition of 1 µM retinoic acid to culture media. Cells were then exposed to DDE or DDT for 48 hours, washed with phosphate-buffered saline, and fixed in 4% paraformaldehyde. Cells were incubated with anti-APP (Sigma Aldrich) and MAP2 (Millipore) primary antibodies, followed by species-appropriate fluorescently labeled secondary antibodies (Jackson Laboratories). Images were captured on a Zeiss Observer D1 microscope (Zeiss Inc) with an X-Cite series 120Q fluorescent illuminator and a Jenoptik camera with

Table 1. Description of the Study Population

Characteristic	Control (n=79)	AD (n=86)
Age, mean (SD), y	70.2 (8.8)	74.1 (8.4)
Sex, No. (%)		
Female	47 (59.5)	47 (54.7)
Male	32 (40.5)	39 (45.3)
Race/ethnicity, No. (%)		
White	69 (87.3)	79 (91.9)
African American	10 (12.7)	7 (8.1)
Family history, No. (%)	30 (38.0)	42 (48.8)
Education, mean (SD), y	15.6 (2.4)	14 (3.5)
MMSE score, mean (SD)	28.9 (1.7)	18.9 (8.1)
APOE, No. (%)		
ε4 positive	28 (35.4)	56 (65.1)
ε4 negative	51 (64.6)	30 (34.9)
Site, No. (%)		
UTSW	54 (68.4)	61 (70.9)
Emory	25 (31.6)	25 (29.1)
DDE nondetects	24 (30.4)	17 (19.8)

Abbreviations: AD, Alzheimer disease; APOE, apolipoprotein E; DDE, dichlorodiphenyldichloroethylene; MMSE, Mini-Mental State Examination; UTSW, University of Texas Southwestern Medical Center.

Figure 1. Serum Levels of Dichlorodiphenyldichloroethylene (DDE)



Serum levels of DDE are elevated in Alzheimer disease (AD). Data were pooled from University of Texas Southwestern Medical Center and Emory University. Levels of DDE are significantly higher in patients with AD (mean [SEM], 2.64 [0.35]) vs control participants (mean [SEM], 0.69 [0.10]; $P < .001$).

ProgRes CapturePro 2.8 software (Jenoptik). Optical density per intensity of fluorescence against APP stain was quantified in individual cells using Image-Pro Plus 7.0 software (Media Cybernetics Inc). Data were calculated as mean (SEM) density/intensity from 3 individual experiments, each performed in triplicate, and data calculated as the percentage of control.

Statistical Analyses

All analyses were conducted with SAS software version 9 (SAS Institute Inc) or Stata version 12. We used nonparametric analysis of variance (Kruskal-Wallis) for bivariate analysis to explore the association between DDE, AD, and other covariates. Correlations between serum and brain levels of DDE were examined using the Pearson correlation coefficient.

Unconditional logistic regression, controlling for age, sex, and location, was used to estimate odds ratios (ORs) and their 95% CIs for the association between serum DDE levels and AD diagnosis in the UTSW and Emory study locations. Generalized estimating equations were used to determine the odds of having AD or decrease in MMSE score per tertile of DDE level in the full study population, controlling for age, sex, race/ethnicity, education, and *APOE* genotype and accounting for location. Confounders were selected on the basis of biological plausibility and 10% change in effect estimate. For samples with nondetectable levels of DDE (n=46), we imputed a value equal to half the limit of detection (0.075 ng/mL), as described by Lubin and colleagues[8] and corrected for cholesterol levels. Regression analysis including the nondetects as zero value did not significantly change the OR estimate. To explore whether the presence of an ε4 allele of *APOE* modified the association between DDE levels and MMSE scores, we either stratified the data by genotype or used an interaction model (DDE**APOE4*) with generalized estimating equations.

Results

Baseline characteristics of the cohort are shown in **Table 1**. There were a total of 165 samples representing 79 control and 86 AD cases. The cohort comprised 94 women and 71 men, with women comprising 60% of the control and 55% of the AD cases. The presence of at least 1 *APOE4* allele was found in 35% of control and 65% of AD cases.

Dichlorodiphenyldichloroethylene (DDE) was detected in 70% of control and 80% of AD cases (Table 1), with mean levels 3.8-fold higher in the serum of AD cases (mean [SEM], 2.64 [0.35] ng/mg cholesterol) compared with control participants (mean [SEM], 0.69 [0.1] ng/mg cholesterol; $P < .001$; **Figure 1**). No other organochlorine pesticide besides DDE was found to be elevated in AD samples compared with control participants (data not shown). The association between serum DDE levels and AD is presented in **Table 2**. Levels of DDE were divided into tertiles, with the nondetects designated at half the limit of detection, and the OR was estimated using generalized estimating equations and corrected for age, sex, race/ethnicity, and location. Compared with the first tertile, the OR for AD diagnosis in the third tertile of DDE level was significantly increased (OR, 4.18; 95% CI, 2.54-5.82; $P < .0001$). The presence of an *APOE* ε4 allele alone was associated with increased AD diagnosis (OR, 3.70; 95% CI, 2.97-4.60; $P < .0001$). However, adjustment for *APOE* genotype did not significantly alter the association between DDE levels and AD diagnosis (Table 2). Furthermore, DDE levels did not differ based on *APOE* genotype (data not shown). To explore the potential influence of nondetects of DDE on AD diagnosis, we performed a sensitivity analysis by comparing the highest tertile of DDE against the nondetects and comparing the highest tertile against the lowest tertile when nondetects were excluded. Similar ORs to our original analysis were observed (eTable 1 in Supplement). Likewise, similar ORs were ob-

Table 2. Odds of AD per Tertile of DDE Distribution

Variable	Serum DDE Level, ng/mg Cholesterol/Tertile of Distribution			*P* Value[a]
	0.09-0.26	0.27-1.64	1.66-18.75	
Odds (95% CI) of AD diagnosis (n=160)				
Adjusted for age, sex, race/ethnicity, and location	1 [Reference]	0.70 (0.19-2.55)	4.18 (2.54-5.82)	<.001
Adjusted for age, sex, race/ethnicity, location, and covariates[b]	1 [Reference]	0.54 (0.13-2.18)	3.40 (1.70-6.82)	<.001

Abbreviations: AD, Alzheimer disease; DDE, dichlorodiphenyldichloroethylene.

[a] *P* value is for the third tertile compared with the first.

[b] Covariates include education and apolipoprotein E status.

Table 3. *APOE4* Polymorphism Modifies the Association Between DDE and MMSE Scores[a]

MMSE	β (95% CI)	*P* Value	*P* Value for Interaction
Independent effects in main effects model			
DDE (3rd tertile vs 1st tertile)	−0.84 (−1.60 to −0.08)	.03	
APOE4	−3.56 (−4.59 to −2.54)	<.0001	
Effect of DDE by *APOE* genotype-stratified model			
APOE4	−1.70 (−3.29 to −0.11)	.04	
APOE2/E3	−0.53 (−0.62 to −0.43)	<.0001	.04
Interaction model			
APOE4	−1.80 (−2.30 to −1.28)	<.0001	
APOE2/3	−1.75 (−3.40 to −0.11)	.04	

Abbreviations: APOE, apolipoprotein E; DDE, dichlorodiphenyldichloroethylene; MMSE, Mini-Mental State Examination.

[a] Controlling for age, sex, race/ethnicity, education, and location in the models.

served when the nondetects were assigned a value of zero (OR, 3.60; 95% CI, 1.23-10.57; $P < .001$).

Mini-Mental State Examination scores were significantly lower in the highest DDE tertile (−0.841; 95% CI, −1.604 to −0.079; $P = .03$) (**Table 3**). Sensitivity analysis demonstrated that excluding nondetects (−1.605; 95% CI, −3.095 to −0.114; $P = .04$) or designating nondetects as zero (−2.628; 95% CI, −5.363 to 0.107; $P = .06$) resulted in similar effect estimates (eTable 2 in Supplement). There was a significant interaction between *APOE* status and DDE levels, where the MMSE score in those with an ε4 allele and DDE levels in the third tertile (OR, −1.6995; 95% CI, −3.293 to −0.106; $P = .04$) was significantly lower (P interaction = .04) compared with those without an ε4 allele (OR, −0.5272; 95% CI, −0.623 to −0.432); $P < .0001$). Serum DDE levels did not differ by genotype, suggesting this is a functional interaction.

To determine the relationship between serum and brain levels of DDE, we measured DDE in 11 matched brain and serum samples from patients with AD collected from the Washington University Alzheimer's Disease Center. For these samples, the serum samples were taken within a year before death and collection of brain tissue. Mean (SEM) serum levels of DDE were 2.69 (0.75) ng/mg cholesterol and were not significantly different from and highly correlated with mean (SEM) brain levels (1.89 [0.46] ng/mg cholesterol; ρ = 0.95; eFigure in Supplement).

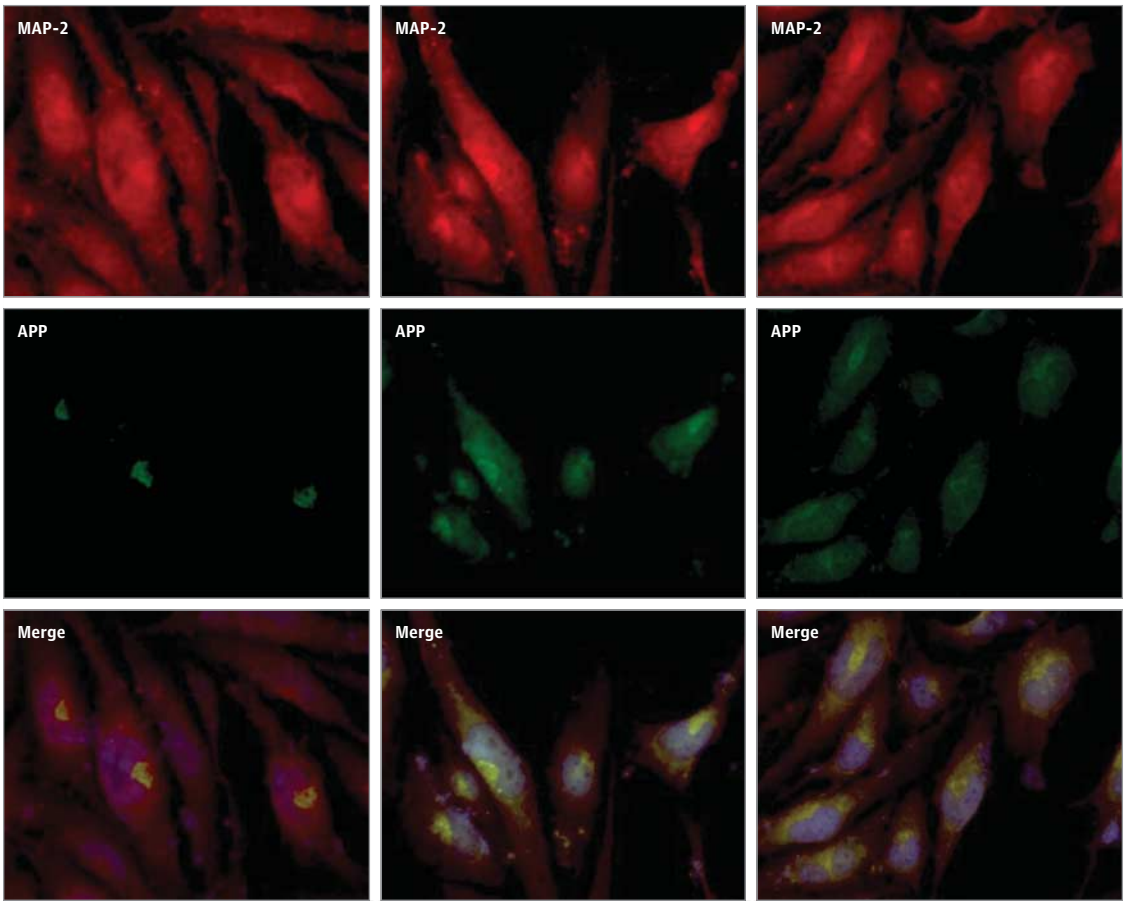
Finally, we sought to determine whether DDE, or its parent compound DDT, has a mechanistic association with AD. A recent study reported that altered network activity in a transgenic AD model, the APP overexpressing mouse, was associated with altered sodium channels.[9] Because sodium channels are the molecular target of DDT and APP overexpression is a causative factor in AD,[10] we hypothesized that DDT or DDE would increase APP levels. To determine this, we exposed cultured neuronal cells and measured the levels of APP, whose genetic overexpression is a risk factor for AD. Exposure of differentiated SY5Y cells to 1-μM DDE or DDT for 48 hours significantly increased APP levels by almost 50% (**Figure 2**).

Discussion

Few studies to date have explored the potential for environmental exposures to contribute to AD. Here, we demonstrated that serum levels of DDE, the metabolite of the organochlorine pesticide DDT, are associated with AD diagnosis and AD severity, as assessed by MMSE. Furthermore, serum DDE levels in the third tertile and the presence of an *APOE* ε4 allele resulted in even greater cognitive impairment. Finally, we demonstrated that concentrations of DDE and its parent compound DDT, similar to those observed in highly exposed individuals in the general population of the United States,[11,12] increased APP levels in cultured neuronal cells. Together, these data identify DDT/DDE exposure as an environmental risk factor for AD.

Dichlorodiphenyltrichloroethane (DDT) was extensively used from the 1940s through 1972 in the United States both in agriculture as a broad-spectrum insecticide and for control of vector-borne diseases including malaria. Although DDT undoubtedly led to major public health victories, the Environmental Protection Agency banned the use of DDT in the United States in 1972 because of concerns regarding its environmental persistence and potential effects on wildlife. At its peak in 1962, production of DDT in the United States was approximately 82 million kg.[13] Currently, several countries around the world continue to use DDT legally and illegally for agricultural purposes, and it is an ingredient in the pesticide Dicofol. Although controversial, the World Health Organization[14,15]

Figure 2. Dichlorodiphenyltrichloroethane (DDT) and Dichlorodiphenyldichloroethylene (DDE) Increase Levels of Amyloid Precursor Protein (APP) in Differentiated SH-SY5Y Cells



SY5Y cells were differentiated with retinoic acid and exposed to 1-μM DDT or DDE for 48 hours. Cells were fixed and stained with antimicrotubule-associated protein–2 (MAP-2) and anti-APP. Original magnification ×20.

supported reintroduction of DDT for malaria eradication in 2006. Thus, there is still significant exposure of human populations.

Levels of DDT and DDE have decreased significantly in the environment over the past 3 decades in the United States. However, DDE is still found in 75% to 80% of serum samples from the Centers for Disease Control and Prevention's cross-sectional National Health and Nutrition Examination Survey.[16] This is likely the result of the exceptionally long half-life of DDE (approximately 8-10 years) and continuing exposure from the import of food from countries where DDT is still used or from legacy contamination of soil and waterways in the United States.[17] The serum DDE concentrations reported here are consistent with those reported by the National Health and Nutrition Examination Survey, with the highest levels in the same range as observed in the 95th percentile.[16] Serum concentrations of DDE are much higher elsewhere in the world where DDT was phased out later or is still used such as Spain and India.[18,19] Importantly, we also found that serum levels are highly correlated with brain levels, which has not been reported before, but is consistent with the high correlation between serum and adipose tissue.[20] Thus, serum levels appear to be an accurate surrogate for DDE levels in the brain.

Although DDT exerts its insecticidal activity through disruption of the nervous system, neither DDT nor DDE are particularly toxic (rat oral LD50s = 113 and 880 mg/kg, respectively).[21] Indeed, administration of DDT or DDE to human individuals for up to 18 months did not cause overt toxicity.[22,23] However, chronic exposure to DDT and DDE has raised concerns about a variety of potential adverse health effects.[13,14,24] Unfortunately, to our knowledge, there are few human studies that have explored the potential neurotoxicity of DDT/DDE. Cueto and colleagues[23] exposed volunteers to 3.5 or 35 mg DDT per day for 12 to 18 months and observed no effects on neurological function. However, 2 other studies found that workers engaged in spraying DDT displayed cognitive dysfunction, although no measurements of DDT or DDE were available for either study.[25,26] Likewise, a large community-based study identified that occupational exposure to organochlorine pesticides was associated with dementia and AD.[5]

One small study reported that DDT was found more often in AD brains (n = 7) compared with control participants (n = 14).[27] Recently, we found an association of serum DDE levels with a diagnosis of AD in a small pilot study, and another study in India found higher serum levels of several organochlorine pesticides, including DDE, in patients with AD.[6,28] Taken together with these studies, our data provide strong support for a role of DDT/DDE in AD. However, we questioned whether this association was mechanistically plausible.

Treatment of SH-SY5Y cells with concentrations of DDT and DDE in the range of concentrations observed in the serum of humans administered 5 to 20 mg of DDT or DDE for 2 to 6 months,[22] in people from an Alabama community with high levels of DDT exposure from industrial dumping of DDT[11] and in people residing in a community near a Superfund site in Maryland,[12] resulted in increased levels of APP, suggesting a possible mechanism for our epidemiological finding.

Our study had a number of strengths including the largest sample size to date for this type of study and the use of well-characterized clinical populations from 2 different geographical locations. The sensitivity analysis ensures that the results were not driven by differences in sampling site or differences in nondetects between cases and control participants. The serum levels of DDE are consistent with those most recently reported by the National Health and Nutrition Examination Survey, suggesting that the cases and control participants were representative of the general population of the United States.[2] Additionally, we provided data demonstrating that serum levels of DDE are highly correlated with brain levels.

There were also limitations to our study. As with our previous study and one other that measured pesticides in the serum of patients with AD, we were limited to studying the persistent organochlorine pesticides.[6,28] Thus, the possibility that other nonpersistent pesticides, such as organophosphates,[5] may contribute to the development of AD in our cohorts cannot be ruled out. A recent study from India found that in addition to DDE, dieldrin and β-hexachlorocyclohexane were elevated in serum samples from patients with AD.[28] However, no detectable levels of dieldrin were found in this study or in more than 200 human serum samples we analyzed in previous studies,[6,7] and β-hexachlorocyclohexane levels significantly decreased in the United States and were not associated with AD in this study.[7] Some patients with AD in our cohort (17 of 86) had nondetectable levels of DDE and control participants were present in the top tertile of DDE levels. This suggests that exposure to DDE may contribute to AD only in a subset of cases, perhaps those with genetic polymorphisms that render them more susceptible to DDT/DDE exposure.

Conclusions

Our findings support epidemiological studies reporting an association of AD with occupational exposure to organochlorine pesticides[5,28,29] and extend them by identifying DDT/DDE as a specific organochlorine pesticide linked to AD in a clinical population from the United States. Indeed, the OR for the association of elevated serum DDE levels with AD is as high as that for *APOE* and the recently reported *TREM2*.[3,30,31] Because elevated DDE levels were associated with significantly worse MMSE performance and exacerbated by the presence of an *APOE* ε4 allele, measurement of serum DDE levels accompanied by *APOE* genotyping might be a useful clinical measure to identify individuals who may be at increased risk for AD. The finding that DDT and DDE increase APP levels in cells provides a mechanistic plausibility to the association between these exposures and AD. If elevation of APP by DDT and/or DDE is confirmed in animal studies and humans, it may provide an avenue for a targeted treatment of individuals with high levels of DDE, such as beta-site APP-cleaving enzyme inhibitors, to prevent cleavage of elevated APP to amyloid-β 42.

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