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AUTOANTIBODIES TO NERVOUS SYSTEM-SPECIFIC PROTEINS ARE ELEVATED IN SERA OF FLIGHT CREW MEMBERS: BIOMARKERS FOR NERVOUS SYSTEM INJURY

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This descriptive study reports the results of assays performed to detect circulating autoantibodies in a panel of 7 proteins associated with the nervous system (NS) in sera of 12 healthy controls and a group of 34 flight crew members including both pilots and attendants who experienced adverse effects after exposure to air emissions sourced to the ventilation system in their aircrafts and subsequently sought medical attention. The proteins selected represent various types of proteins present in nerve cells that are affected by neuronal degeneration. In the sera samples from flight crew members and healthy controls, immunoglobin (IgG) was measured using Western blotting against neurofilament triplet proteins (NFP), tubulin, microtubule-associated tau proteins (tau), microtubule-associated protein-2 (MAP-2), myelin basic protein (MBP), glial fibrillary acidic protein (GFAP), and glial S100B protein. Significant elevation in levels of circulating IgG-class autoantibodies in flight crew members was found. A symptom-free pilot was sampled before symptoms and then again afterward. This pilot developed clinical problems after flying for 45 h in 10 d. Significant increases in autoantibodies were noted to most of the tested proteins in the serum of this pilot after exposure to air emissions. The levels of autoantibodies rose with worsening of his condition compared to the serum sample collected prior to exposure. After cessation of flying for a year, this pilot's clinical condition improved, and eventually he recovered and his serum autoantibodies against nervous system proteins decreased. The case study with this pilot demonstrates a temporal relationship between exposure to air emissions, clinical condition, and level of serum autoantibodies to nervous system-specific proteins. Overall, these results suggest the possible development of neuronal injury and gliosis in flight crew members anecdotally exposed to cabin air emissions containing organophosphates. Thus, increased circulating serum autoantibodies resulting from neuronal damage may be used as biomarkers for chemical-induced CNS injury.

Airline crew and passengers may be exposed to air emissions including engine oil contaminants, such as gaseous, vapor, and particulate constituents of pyrolyzed engine oil in the unfiltered ventilation air supply that is extracted from either the aircraft engines or auxiliary power unit (APU). Such exposure has been associated with a variety of symptoms related to central nervous system (CNS) dysfunction, as well as effects on the gastrointestinal, respiratory, dermal, and perhaps immune systems (Cox and Michaelis, 2002; Winder

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and Balouet, 2002; Winder 2006; Murawski and Supplee, 2008). Numerous reports over the past 50 years documented neurological complaints among commercial and military cabin crews after exposure to air emissions in aircraft (Kitzes, 1956; Montgomery et al., 1977; Carletti et al., 2011). This condition is sometimes referred to as aerotoxic syndrome (Winder et al., 2002).

Ross (2008) reported the outcome of psychological assessments of 27 self-reported pilots who noted CNS symptoms. These symptoms were postulated to have resulted from exposure to the organophosphates (OP) compounds present in engine oil and hydraulic fluid. Organophosphates exert three distinct neurotoxic actions (1) Cholinergic neurotoxicity resulting from inhibition of acetylcholinesterase (AChE) and over-stimulation of muscarinic and nicotinic acetylcholine (ACh) receptors with subsequent development of cholinergic-associated toxicity (Abou-Donia, 2003); (2) Organophosphorus ester-induced delayed neurotoxicity (OPIDN) that is a central–peripheral axonopathy, characterized by primary Wallerian-type axonal degeneration of the CNS and peripheral nervous system (PNS), followed by secondary demyelination (Smith et al., 1930; Abou-Donia, 1981, 1995; Abou-Donia and Lapadula, 1990). The clinical picture for OPIDN is manifested initially by mild sensory disturbances, ataxia, weakness, muscle fatigue, and twitching, which may progress to paralysis. Improvement in OPIDN is slow and may require years and recovery may not be possible. (3) Organophosphorus ester-induced chronic neurotoxicity (OPICN) is characterized by long-term neurological and neurobehavioral deficits accompanied by brain neuronal cell death (Abou-Donia, 2003). This long-lasting effect results primarily from injury to the CNS.

Engine oil contains a mixture of tricresyl phosphate isomers (TCP, 2–6% by weight). TCP isomers are produced by the reaction of cresols and phosphorus oxychloride. The cresol may be a mixture of three isomers: *ortho, meta,* and *para*. By regulation, the total tri-*ortho*-cresyl phosphate (TOCP) content must be less than 0.2% of the total TCP (Mattie et al., 1993). For more than a century, TOCP was shown to induce OPIDN in humans and experimental animals (Smith et al., 1930; Abou-Donia, 1981; Suwita and Abou-Donia, 1990). Ingestion studies have associated the neurotoxicity attributed to TCP mixtures with its TOCP content (Craig and Barth, 1999; Mackerer et al., 1999; Freudenthal et al., 1993). Inhalation neurotoxicity due to pyrolyzed engine oil/TCP was reported to be higher than expected (Lipscomb et al., 1995).

Hydraulic fluid is composed of a mixture of tributyl phosphate (TBP), dibutyl phenyl phosphate (DPP), and butyl diphenyl phosphate (BDP), all of which were detected in air emissions on aircrafts (van Netten and Leung, 2001; Winder et al., 2002; De Nola et al., 2008). Organophosphate components of turbo oil and hydraulic oil produce relatively low cholinergic neurotoxicity. Vapors of TCP and TBP produced neurotoxicity in rats (Lipscomb et al., 1995). Workers exposed to 15 mg/m^3 air containing TBP complained of nausea and headaches (ACGIH, 1986). On the other hand, some of these chemicals are capable of producing OPIDN and/or OPICN. Tri-aryl phosphates produced OPIDN in test animals only following large doses (Weiner and Jortner, 1999).

The severity of symptoms reported after an aircraft exposure is enhanced by combined exposure to other toxicants, many of which are undefined, but that include OP pyrolysis products (Abou-Donia et al., 1996). Trimethylolpropane phosphate (TMPP), one of these components, is a potent neurotoxicant that is formed when engine oil reacts with TCP within the aircraft engine (Rubey et al., 1996). Other airborne contaminants include carbon monoxide (CO) and carbon dioxide (CO_2), acrolein, ozone, volatile organic compounds (VOC), and particulates that were detected in the airplane environment (van Netten and Leung, 2001; Rayman, 2002; Winder and Balouet, 2002).

Conventional clinical neuroimaging is relatively insensitive in detecting neuronal and glial injury following chemical-induced brain injury. Therefore, clinically available biomarkers for

neuronal injury are essential in the diagnosis and understanding of the temporal progression of the injury (Jauch et al., 2006). The use of serum biomarkers, such as cytoskeletal proteins, in diagnosing brain injury has dramatically improved care for patients with traumatic brain injury (TBI; Zurek and Fedora 2012). Protein fragments from injured neuronal and glial cells are found in cerebral spinal fluid (CSF), but are unlikely to survive long enough in blood to be practical markers because of their short half-lives.

The present descriptive study of flight crew members was undertaken to test the hypothesis that following brain injury, neuronal and glial proteins that are normally present in the CNS and PNS leak from injured areas through the damaged blood–brain barrier (BBB; Abdel-Rahman et al., 2002) and from degenerated peripheral nerves. Once in circulation, these proteins act as autoantigens and react with B lymphocytes that are normally produced in bone marrow to form autoantibodies also known as immunoglobin (IgG), or "memories" of these specific proteins. This test converts the short-lived nervous-system-specific proteins in the serum into long-term biomarkers for neurologic damage.

The presence of circulating IgG-class autoantibodies in sera from a sample of healthy controls and flight crew members was assayed against cytoskeletal proteins associated with (1) neurogenesis, that is, neurofilament triplet proteins (NFP), tubulin, tau, and microtubule-associated protein-2 (MAP-2); (2) myelinogenesis, that is, myelin basic protein (MBP); and (3) astrogliogenesis, that is, glial fibrillary acidic protein (GFAP) and glial S100B protein. Both GFAP and S100B are secreted by astrocytes and are the only two antigens studied not present in the PNS and therefore reflect effects on only the CNS. Autoantibodies against these neuronal and glial proteins were reported to monitor brain injury and correlated with human brain disorders (Ingram et al., 1974; Hoshi et al., 1988; Lee et al., 1988; Ahlsen et al., 1993; Dotevall et al., 1999; Spillantini and Goedert, 1998). It is also postulated that serum concentrations of autoantibodies against these proteins are (1) related to the degree of neurological deficits and (2) associated with functional outcomes.

MATERIALS AND METHODS

Materials

The sources of proteins were: NFP (mass, NFL, 70, NFM, 160, NFH, 200 kD; bovine spinal cord), tau protein (mass, 45–68 kD; human), MAP-2 (mass, 300 kD; bovine serum), tubulin (mass, 55 kD; bovine brain), and MBP (mass, 33 kD; human brain), from Sigma-Aldrich (Saint Louis, MO), GFAP (mass 52 kD; human) from Biotrend Chemikalien GmbH (Cologne, Germany), and S100B (mass 9–14 kD; human brain) from American Qualex International, Inc. (San Clemente, CA). Horseradish peroxidase-conjugated goat anti-human IgG, and enhanced chemiluminescence reagent were obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Sodium dodecyl sulfate (SDS) gels, 2–20% gradient (8 × 8), and tris-glycine at 15 m*M* were obtained from Invitrogen (Carlsbad, CA). All other materials were purchased from Amersham.

Western Blot Assay

To screen for the presence of autoantibodies against the battery of proteins, the proteins were separated using Western blot assay. Each serum sample was analyzed in triplicate. All proteins were loaded at 10 ng/lane except for albumin, which was loaded at 100 ng/lane. Proteins were denatured and electrophoresed in SDS–polyacrylamide gel electrophoresis (PAGE) (4% to 20% gradient) purchased from Invitrogen (Carlsbad, CA). One gel was used for each serum sample. The proteins were transferred into polyvinylidene fluoride (PVDF) membranes (Amersham). Nonspecific binding sites were blocked with Tris-buffered saline-Tween (TBST) (40 m*M* Tris [pH 7.6], 300 m*M* NaCl, and 0.1% Tween 20) containing 5% nonfat dry milk for 1 h at 22°C. Membranes were incubated with

serum samples at 1:100 dilutions in TBST with 3% nonfat dry milk overnight at 4°C. After 5 washes in TBST, the membranes were incubated in a 1:2000 dilution of horseradish peroxidase-conjugated goat anti-human IgG (Amersham). The membranes were developed by enhanced chemiluminescence using the manufacturer's (Amersham) protocol and a Typhoon 8600 variable mode imager. The signal intensity was quantified using Bio-Rad image analysis software (Hercules, California). All tests were performed with the investigators masked to diagnosis.

Specificity of Sera Autoantibodies

To check the specificity of the sera autoantibody, a peptide/antigen competition assay was performed. In this assay sera were mixed with the target protein or peptide with the objective of eliminating the binding of the autoantibody to the protein in the serum. Briefly, 50 μl of serum from random three subjects was mixed with or without 2 μg of tau, MAP, or MBP in 100 μl PBS and rotated overnight at 4°C. The serum/protein mix was centrifuged at 12,000 × g to pellet any immune complexes. The supernatant was then removed and used for Western blotting.

Calculations

Optical density measurement for subjects and healthy controls was divided by serum albumin density concentration; this value for each subject was normalized to controls and expressed as fold-change from healthy controls. Thus, the results are expressed as mean values of triplicate assays of optical density arbitrary units normalized to albumin optical density as fold of healthy controls.

Controls and Subjects

Under a protocol approved by the Institutional Review Board (IRB) at Duke University Medical Center, sera were collected from 12 healthy controls, referred to as controls below, and 34 flight crew members, referred to as subjects in the following, who experienced symptoms and sought medical attention, approximately 2–4 wk after last exposure to air emissions and onset of symptoms. The controls were of both genders, had no connection with the aviation industry, were age-matched with the subjects, and did not report exposures to air emissions or any neurological symptoms. The subjects were pilots and flight attendants from commercial airlines, with cumulative flying hours ranging from 4,000 to 16,500. The subjects in this study were seen by physicians and self-reported their complaints in questionnaires. Individuals reported that that they did not have any known neurological symptoms previously, and provided consent to participate in this study. Subjects included both genders and ranged from 31 to 63 years in age. Blood was drawn at the time of diagnosis. Sera were stored at −70°C.

CASE STUDY SUBJECT

A healthy 50-year-old male professional airline pilot, with 25 yr and approximately 15,000 cumulative hours of flying, had been on vacation for 2 mo and did not fly during that period as a passenger. Table 1 shows flying activity, serum sampling, and clinical condition of the pilot. In total, four serum samples were taken in this study. The pilot was symptom free prior to the flight in question, felt "fit to fly," and provided consent to participate in the study and the first serum sample number 1 at time "0" before flying.

He then embarked on a set of flights totaling 45 h in 10 d, during which he reported episodes of air emissions. Average concentration of TCP isomers in cabin air during these flights was 0.65 ng/m^3 (Spectrex PAS 500 + SKC 106 Chromosorb test tube); dust on a sample tissue from the aircraft floor yielded 1270 ng TCP isomers. (TNO, Utrecht, The Netherlands). His only complaint at that time was bad memory. He gave a serum sample number 2 at 12 days.

He continued to fly for 9 mo despite worsening of his memory deficits. A month later, he complained of a sudden deafness

TABLE 1. Flying Activity, Serum Sampling, and Clinical Condition of the Case Study Pilot

Time	Serum sample/Clinical condition	Symptoms
0	1	No symptoms, able to fly
12 d	2, Onset of memory deficits	After the pilot had flown 45 h total over a period of 10 d. Exposure to air emission; average concentration of TCP isomers during these flights was in cabin air 0.65 ng/m^3 and in cabin dust 1270 ng of TCP isomers. The only complaint was bad memory.
After 9 mo	Worsening of memory deficits.	More symptoms: Nine months later, after having flown normal duties during the following months, memory deficits became worse.
After 10 mo	Deafness, vertigo	After another month (total of 10 mo): sudden deafness and vertigo on one side. He was grounded as a result.
16 mo	3, Severe symptoms	After a period of 6 mo, with extended spells of nausea, vomiting, nystagmus, and loss of equilibrium, neurosurgery was suggested (not done) on the affected inner ear leading to a permanent state of "unfit to fly.". Full-fledged symptoms.
17.5 mo	Onset of recovery	After 1.5 months (at 17.5 months of first sample) of treatment, clinical improvement was noted; vomiting stopped and he could walk upright again, without falling over to one side.
21 mo	4, Recovery	He became fit to fly and regained his Airline Transport Pilot License Medical Certificate and is still able to perform his flying duties and remain free of symptoms, while on a regular daily treatment. Recovery.

and vertigo on one side. As a result, the pilot was grounded, 10 mo after giving the first serum sample. During the following 6 mo, with extended spells of nausea, vomiting, nystagmus and loss of equilibrium, and inner ear problems, he was given a permanent state of "unfit to fly". He gave the third serum sample at "16 months" after the first serum sample with severe symptoms. With medical care, vomiting stopped 1.5 mo later; he could walk upright again, without falling over to one side. This was followed by recovery, becoming fit to fly, and regaining his Airline Transport Pilot License Medical Certificate. He is still able to perform his flying duties and remains free of symptoms while still remaining under medical care. He gave the fourth serum sample at 21 mo.

Statistics

Grouped data are reported as mean ± standard error. The values from subjects were compared to controls using a paired *t*-test. Mean values for autoantibodies from the subject group were compared to controls using two-way analysis of variance (ANOVA; SigmaStat, Systat Software). A p value $<.05$ was set as the criterion for significance.

RESULTS

The results of this descriptive study report an association between self-reported neurologic deficits and levels of autoantibodies against neuron- and glia-specific proteins in sera from a sample of flight crew members (pilots and flight attendants).

Specificity of Sera Autoantibodies

To detect the presence and specificity of autoantibodies in sera samples, a study was initially carried out to demonstrate the specificity of serum autoantibodies to the tested target proteins: tau, MAP-2, or MBP; the results are presented in Figures 1A–1E. Human serum (unbound) from a subject with neurological deficits showed increased band signal in tau, MAP-2, and MBP (Figure 1A). When normal rabbit serum was used no band signals were detected in tau, MAP-2, and MBP (Figure 1B). The serum bound to tau eliminated the tau band in the Western blot, while the band of MAP-2 or MBP was present and not affected (Figure 1C). The serum bound to MAP-2 eliminated the MAP-2 band in the Western blot while the band of tau or MBP was present (Figure 1D). The serum bound to MBP

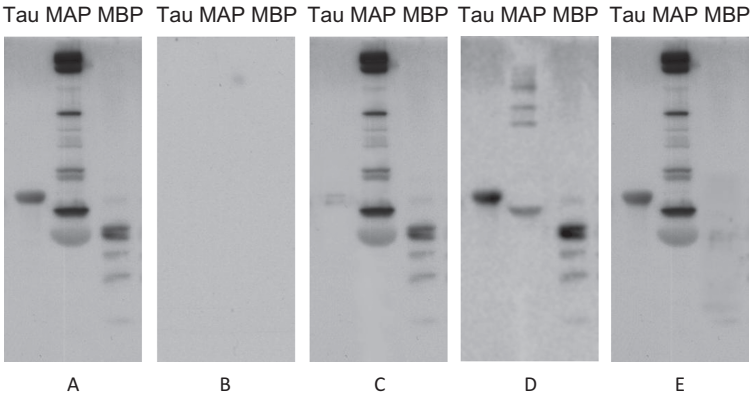


FIGURE 1. Characterization of presence and specificity of autoantibody in human serum samples. (A) Western blot of unbound serum shows band signals in tau, MAP, and MBP. (B) Normal rabbit serum shows no band signals detected. (C) Serum bound to tau shows no band signal with tau protein. (D) Serum bound to MAP shows no band signal with MAP protein. (E) Serum bound to MBP shows no band signal with MBP.

eliminated the MBP band in the Western blot while the bands of tau and MAP-2 were present (Figure 1E). These results indicate that each autoantibody in the serum was specifically neutralized by its target protein in sera samples and was no longer available to bind to the epitope present in the protein on the Western blot. This confirmed that the assay used in this study was specific and reliably determined autoantibodies against tested proteins in sera samples.

Self-Reported Clinical Symptoms by Subjects

The subjects reported that 2–4 wk after exposure to air emissions they went to physicians with initial symptoms related to cholinergic toxicity; however, the individuals continued to have chronic symptoms. Figure 2 shows the self-reported complaints of the airline crews. The hallmark of their symptoms consisted of the following three complaints, which were reported by more than half of the subjects: memory deficits, headaches, and fatigue. The frequencies of these three symptoms were higher than the following complaints that were reported by approximately one-third of the subjects: muscle weakness/pain, imbalance, respiratory problems, and tingling hands and feet. Approximately 20% of subjects reported the following complaints: dizziness, vision problems, anxiety, confusion, anger/aggression, and ear ringing. Between 12% and 15% of subjects reported depression, tachycardia, slurred speech, nausea, and urinary frequency. The following complaints were cited by 9% of subjects: speech/spelling difficulties, dyslexia, fear/panic, tremors, and skin problems. Six percent of subjects complained of stress, stomach pain, chemical sensitivity, and sexual impairment. Only 3% of subjects reported a single complaint of: falling asleep, hair loss, *Herpes* after flight, mood swinging, temperature control, diarrhea, or high blood pressure. Many of theses symptoms persisted long after exposure.

Autoantibodies in Sera from Subjects and Controls

Increased levels of these autoantibodies were detected in subjects who reported symptoms related to their exposure to air emissions in aircrafts compared to healthy controls. Table 2 and Figure 3 present the levels of the sera-circulating IgG-class autoantibodies against neuronal and glial proteins from controls and flight crew members. Sera from healthy controls had no or low levels of circulating autoantibodies to nervous system proteins. In contrast, there were significant increases in levels of autoantibodies of all tested proteins in sera of the subjects compared to healthy controls. Mean levels of autoantibodies in the subjects and controls are presented in Table 2 and

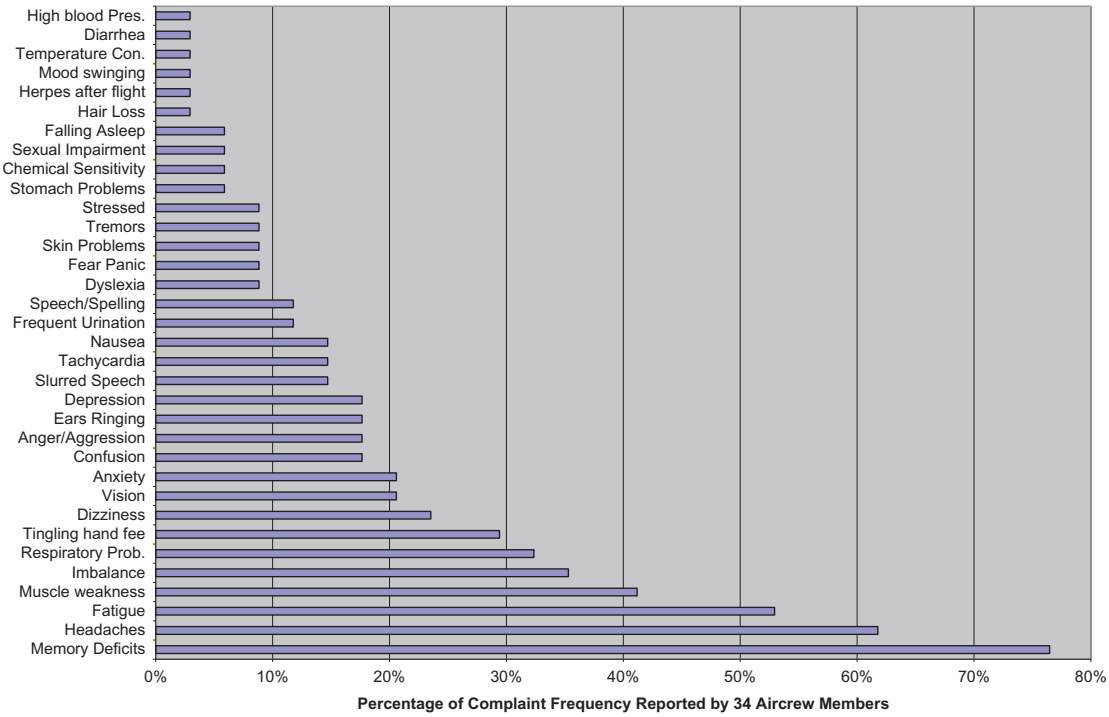


FIGURE 2. Frequency of complaints reported by 34 flight crew members (color figure available online).

TABLE 2. Sera Autoantibodies Against Neuronal and Glial Proteins From Subjects and Controls

Autoantibodies to proteins	Subjects	Controls	Foldsincrease
Microtubule-associated protein-2 (MAP-2)	7.22 ± 0.74^{b}	1.18 ± 0.20	6.12
Tubulin	6.20 ± 0.77^{a}	1.02 ± 0.21	6.08
Myelin basic protein (MBP)	5.65 ± 0.72^{a}	0.69 ± 0.18	8.19
Tau Proteins	4.18 ± 0.48^{a}	0.83 ± 0.14	5.04
Glial fibrillary acidic protein (GFAP)	3.41 ± 0.53^{a}	0.61 ± 0.17	5.59
Neurofilament proteins (NFP)	3.10 ± 0.43^{a}	0.52 ± 0.11	5.96
S100B	0.45 ± 0.07^{a}	0.24 ± 0.05	1.88

Note. Autoantibodies are expressed as optical density arbitrary units normalized to albumin in each human serum, and represent the mean values ± SE of triplicate assays.

[a]Significant at $p < .001$.

were in descending order: MAP-2 > tubulin > MBP > tau > GFAP > NFP > S100B.

CASE STUDY SUBJECT

Figure 4 presents the results of Western immunoblots of autoantibodies against the tested nervous system proteins in sera of a control and case-study pilot before and after exposure to cockpit air emissions: (A) control, (B) the symptoms-free pilot at time "0," (C) the pilot after 12 h of flying after exposure to air emission, (D) the pilot after 16 mo from first serum sample with severe symptoms, and (E) the pilot at 21 months, after the first serum sample following recovery.

Table 3 quantifies the results of Western immunoblots of autoantibodies to nervous-system proteins for all four serum samples. Results show significant increases in autoantibodies against nervous-system-specific proteins, 12 d after flying and exposure to air emissions, in the following descending order: tubulin > MBP > MAP-2 > tau > S-100 > GFAP. Data also demonstrate that after 16 mo of flying with severe symptoms compared to

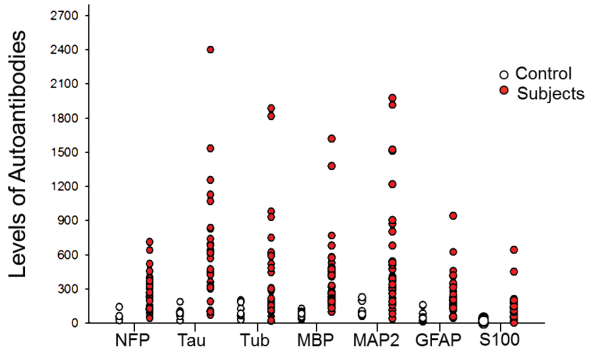


FIGURE 3. The levels of the sera circulating Ig-G-class autoantibodies against neuronal and glial proteins from controls and flight crew members. The results are expressed as means of optical density arbitrary units normalized to albumin optical density from each serum as fold of healthy controls and represent means ± SE of triplicate assays (color figure available online).

levels at "0" serum, the pilot's serum still contained higher levels of autoantibodies against the following nervous-system-specific proteins: S-100 > tubulin > MBP > GFAP. In contrast, after recovery, only autoantibodies against tubulin and MBP were still higher than before exposure levels, but at lower levels in serum taken at 21 mo than in earlier serum samples. It is noteworthy that when the original two sera samples, stored in a −70°C freezer, were assayed 2 yr after their collection, there was little change in their levels of autoantibodies.

DISCUSSION

Although this study was designed to be descriptive rather than statistically powered, statistically significant elevations in serum autoantibodies to neuronal and glial proteins were detected in sera of the subjects (pilots and attendants) who were exposed to air emissions during their flights and subsequently

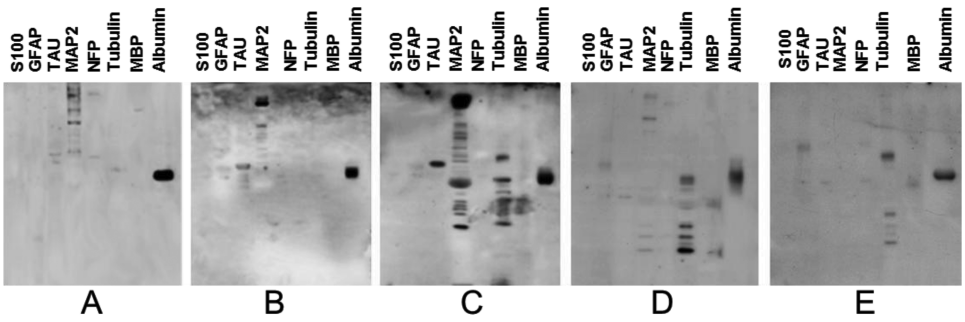


FIGURE 4. Western immunoblots of autoantibodies against tested nervous system proteins in sera of control and case-study pilot before and after exposure to cockpit air emissions: (A) control, (B) the pilot serum before flying, time "0," (C) the pilot after "12 days" of flying, (D) the subject "16 months" after the first sample, and (E) the subject "21 months" after the first sample.

TABLE 3. Sera Autoantibodies Against Neuronal and Glial Proteins From Case-Study Pilot Before and After Exposure[a]

Proteins	Time "0" before exposure, level[a]	12 d after exposure		16 mo after exposure		21 mo after exposure	
		Level[a]	Change[b]	Level[a]	Change[b]	Level[a]	Change[b]
MAP-2	3.03	16.7[c]	5.33	2.05	0.60	1.28	0.42
TAU	1.09	5.56[c]	5.10	1.21	1.11	0.94	0.86
Tubulin	0.34	2.86[c]	17.24	7.67[c]	22.00	3.78[c]	11.12
MBP	0.33	2.85[c]	8.64	3.45[c]	10.45	2.33[c]	7.06
NFP	0.35	0.20	0.57	1.01[c]	2.88	0.68	1.94
GFAP	0.32	0.72	2.25	0.93[c]	2.90	0.43	1.30
S-100B	0.03	0.15	5.00	0.82[c]	27.00	0.04	1.33

[a]Autoantibodies are expressed as optical density arbitrary units normalized to albumin in each human serum, and represent the mean values of triplicate assays.

[b]Fold change from values before exposure at time "0."

[c]Values showed significance at $p < .001$.

developed neurologic symptoms compared to healthy controls. The early symptoms such as shortness of breath, irritation of eye, nose, and throat, headache, nausea, dizziness, stomach cramping, and muscle weakness reported by the subjects are consistent with OP-induced cholinergic neurotoxicity. Chronic symptoms included headache, memory impairment, vision changes, vertigo, neuromuscular pain, fatigue, and tremors and are in agreement with OPIDN and/or OPICN (Abou-Donia, 2003). The results for healthy controls are in agreement with previous finding in healthy individuals of no or low-level quantities of circulating serum autoantibodies to neuronal and glial proteins (Ingram et al., 1974), suggesting that controls had no or little exposure to OP. In contrast, marked increases in autoantibodies to nervous-system-specific proteins were observed in pilots and attendants compared to controls. These elevations in the autoantibodies were consistent with their neurological complaints and are listed in descending order: MBP, MAP-2, tubulin, NFP, GFAP, tau, and S100B.

Although preexposure serum samples were unavailable, it is unlikely that a single exposure or an exposure for a few days before sampling would yield the results of IgG autoantibodies presented in Figure 3. The differences in autoantibody concentrations reported are most likely the result of nervous-system damage, rather than the product of time between exposure and sampling. The development of autoantibodies may be likely to result from release of nervous-system-specific proteins produced by tissue injury following multiple exposures to air emissions, over a long time. Production of autoantibodies in flight-crew personnel seems to be an ongoing process over an extended period of exposure to air emissions before onset of symptoms and seeking medical treatment. Initially, little IgG is formed; cells producing antibodies need to go through class-switching before producing IgG (Pollard et al., 2010). Following immunization (deliberate or, in this case, accidental), IgG titers rise over time. Therefore, it is concluded that recent exposure of subjects to air emissions produced neurodegeneration above the threshold level to induce neurologic deficits and release of nervous system-specific proteins leading to formation of autoantibodies and release into circulation.

Increased levels of autoantibodies circulating in sera of the subjects who reported symptoms related to their exposure to air emissions in aircrafts compared to healthy controls followed the pattern given next:

- Autoantibodies to MBP, an abundant membrane proteolipid produced by oligodendroglia in the CNS and Schwann cells in the PNS (Jauch et al., 2006), showed the highest level in subjects compared to controls. These findings correlate with demyelination following axonal degeneration in both CNS and PNS that was induced by exposure to OPs (Abou-Donia, 1981).
- MAP-2 proteins are present almost exclusively in the somatodendritic compartments on neurons and these exhibited high levels of autoantibodies to MAP-2 in the subjects. The microtubule-associated proteins, MAP-2 and tau, function in promoting polymerization and stabilization of microtubules in axons, cross bridge neurofilaments, and microtubules connecting themselves to each other. These functions maintain axonal transport (Hoshi et al., 1988).
- Autoantibodies to tubulin which is present in virtually all eukaryotic cells in addition to neurons exhibited high levels in flight crew members. Microtubules are composed of α- and β-tubulin that constitute approximately 10% of total brain proteins, and are responsible for axonal migration and longitudinal growth and are involved in axonal transport (Laferrière et al., 1997; Damodaran et al., 2009, 2011).
- Increased autoantibodies to neurofilaments in subjects are in agreement with the finding that their destruction is involved in neurodegeneration (Jensen et al., 1992, Brady, 1993). NFP subunits are the major component of the neuronal cytoskeleton, accounting for 85% of total protein in neuronal cell (Fuchs and Cleveland, 1998); NFP consist of three

polypeptides: low-molecular-weight (NF-l), middle or medium-molecular weight protein (NF-M), and outer or high-molecular weight protein (NF-H) (Lee et al., 1988). NF subunits regulate axonal caliber; neurofilaments therefore affect both axonal transport and neuronal function (Tagliaferro et al., 2005).

- The levels of autoantibodies to tau proteins observed in subjects were less than those against MAP-2. Tau proteins are cytoskeletal proteins localized primarily in the axonal compartment in neuronal cells, and are composed of six isoforms. Tau proteins (1) bind to axonal microtubules to form microtubule bundles, (2) are more abundant in white matter than gray matter, (3) are elevated in the cerebrospinal fluid (CSF) and serum following traumatic brain injury (TBI) (Liliang et al., 2010), and (4) are used for diagnosis of Alzheimer's disease (Shiiya et al., 2004).
- Autoantibodies to astrocytic proteins—GFAP and S-100—were significantly elevated in flight crew members compared to controls, although less than neuronal proteins levels. Increased levels of autoantibodies in serum to GFAP are associated with gliosis. GFAP is released from astrocytes after CNS injury, cellular disintegration, and degradation of the cytoskeleton (Kovesdi et al., 2010). Elevated levels of GFAP are regarded as a nonspecific biomarker for brain injury in several CNS diseases including dementia (Eng, and Ghirnikar, 1994), brain infarction (Aurell, et al., 1991), Lyme neuroborreliosis (Dotevall et al., 1999), and neuropsychiatric disorders (Ahlsen et al., 1993).
- S100B autoantibodies were elevated in sera of subjects. S-100B interacts with and stabilizes microtubule-associated proteins, tau, and MAP-2. S100B is labeled as a marker of generalized BBB dysfunction (Kapural et al., 2002). Traumatic acute injury that results in extensive destruction of astrocytes leads to a significant release (50- to 100-fold) of S100B into serum, whereas S100B levels in psychiatric disorders were approximately threefold higher in patients compared to controls (Arolt et al., 2003), correlating with a neuroprotective capacity. Such findings were documented in cases of dementia, particularly Alzheimer's disease (Griffin et al., 1989), schizophrenia and major depression (Grabe et al., 2001), and mania (Machado-Vieira et al. 2002).

The case study of the one pilot demonstrates the temporal relationship between exposure to the airliner's air emissions, development of neurologic deficits, and increased serum autoantibodies against nervous system specific proteins. The timing of when serum samples were taken relative to exposure is important to establish a plausible link between exposure, damage, and presence of IgG circulating autoantibodies. It is not likely that blood samples taken within days of exposure would contain IgG circulating autoantibodies to neoantigens at the levels found in this study. The results of the pilot's serum exhibit high levels of autoantibodies to nervous system proteins 12 d after exposure to air emissions. At that time he exhibited only memory deficits, suggesting that autoantibodies production was an ongoing process and resulted from the breakdown of nervous system proteins over a long period of time during the pilot's 25 yr and 15,000 of cumulative flying hours. Further, high levels of IgG autoantibodies to nervous-system-specific proteins were still detected in serum samples taken 16 mo after the first serum sample. At that time he exhibited severe symptoms that were related to nervous-system damage and subsequent autoantibodies formation. His condition began to improve 17.5 mo after the first serum sample and was completely recovered by 21 mo when the last serum sample was taken. Autoantibodies against the nervous system proteins at 21 months were lower than found in previous serum samples. The results from this case study subject indicate that improvement in the clinical condition, and even recovery, are possible within 21 mo following nervous system injury. This improvement may be followed by assaying the subject's serum autoantibodies levels.

The clinical improvement observed in the pilot may be attributed to (1) regeneration of

the PNS, (2) regeneration of the GFAP- and S100B-forming astrocytes, and (3) other CNS neuronal cells assuming the function of damaged cells (Abou-Donia 1981, 2003). Although clinical improvement was observed in the pilot's CNS injury, the loss of neurons may have rendered his CNS more prone to damage following any future chemical exposure, including air emissions. Improvement in the pilot's health was accompanied by the return of most autoantibodies to nervous system proteins to their preexposure levels, except for those against tubulin and MBP. These changes suggest some neuronal recovery consistent with no further chemical exposure. The persistence of autoantibodies against tubulin may be related to the presence of tubulin in all eukaryotic cells in addition to neurons. The observation that autoantibodies against tubulin and MBP were less than right after exposure suggests that axonal degeneration and demyelination may have peaked and were not continuing.

It is possible that a low-level, symptom-free exposure might result in similar patterns of autoantibodies. If that does occur, then a numerical rise in autoantibodies may only signal exposure but not actual damage, or it may reflect ongoing chronic exposure that results in neurodegeneration below the threshold where neurologic deficits occur. This observation is consistent with the presence of autoantibodies against some nervous-system proteins in the pilot's serum prior to episodes of toxic air emissions and absence of observable neurologic deficits. This finding is also in agreement with detection of low-level exposures to TCP on aircraft that do not result in overt acute symptoms (IEH, 2011). Of 100 flights studied on 4 aircraft, detectable airborne levels of these chemicals were found for TOCP on 14, TCP (non-TOCP, multiple isomers) on 23, and TBP on 73. This observation is also in agreement with recent detection of plasma butyrylcholinesterase (BChE)-phosphorylated adduct with cresyl saligenin phosphate (a biomarker for TOCP exposure) in jet airplane passengers who did not have clinically overt neurologic symptoms (Liyasova et al., 2011). Air samples collected from aviation mechanics areas contained TCP and tri-*n*-butyl phosphate (Solbu et at, 2007). The presence of TCP and TOCP in cabin air on commercial and military aircraft was confirmed by in-flight air sampling in the absence of overt adverse symptoms (van Nettten, 1998; 2009; IEH, 2011).

The present results are consistent with the development of neuronal damage and gliosis in pilots and attendants. The fivefold increase in autoantibodies against S100B is in agreement with development of a recent brain injury followed by long-term neurological deficits (Arolt et al., 2003). The temporal rise in both autoantibodies and neurologic deficits suggests a pathophysiologic connection between exposure to cabin air emissions, neuronal degeneration, and reported complaints. Increased numbers of autoantibodies against neurofilament, tau, tubulin, and myelin basic proteins, which are biomarkers for axonal degeneration, in brain regions such as the cerebral cortex account for motor and sensory abnormalities, ataxia, weakness, and loss of strength reported by the subjects. Damage to the hippocampal circuitry leads to learning and memory deficits. Neuronal degeneration of the limbic system and central motor system associated with mood, judgment, emotion, posture, locomotion, and skilled movements results in psychiatric disorders. Autoantibodies against GFAP are elevated in CNS diseases including dementia and brain infarction. Finally, the elevation in S100B levels in flight crew members is consistent with psychiatric disorders occurrence.

The present results are in agreement with a study that detected autoantibodies to NFP in a child who became quadriplegic following exposure to TOCP (Abou-Donia and Garretson, 2000) and with another investigation that detected increases in myelin of PNS and CNS in personnel chronically exposed to chlorpyrifos (Thrasher et al., 2002). In addition, autoantibodies against NFP, GFAP, and MBP were detected in the serum of a 16-yr-old boy poisoned with the OP insecticide methamidophos who developed OPIDN (McConnell et al., 1999). Autoantibodies to NFP, MBP, and GFAP were elevated in hens that developed OPIDN following exposure to phenyl saligenin

phosphate, the active neurotoxic metabolite of TOCP (El-Fawal et al., 1999).

Since the recognition of TOCP-induced neurotoxicity, designated as OPIDN in 1981 (Abou-Donia, 1981), numerous studies have been carried out to elucidate mechanisms of action (MOA). Because early studies on the involvement of esterases, including AChE (Bloch and Hottinger, 1943), BChE (Earl and Thompson, 1952), and neurotoxicity target esterase (NTE; Johnson 1969), did not enhance the understanding of MOA of OPIDN, studies in the past 30 years focused on kinases. The results of these studies identified a major role for Ca^{2+}-calmodulin kinase II (CaMKII) in the pathogenesis of OPIDN. An early event in OPIDN is increased Ca^{2+} concentration in neuronal mitochondria of hen spinal cord (LoPachin et al., 1988). This is followed by enhanced autophosphorylation (Patton et al., 1983, 1985, 1986), by elevated CaMKII activity (Lapadula et al., 1991, 1992; Abou-Donia et al., 1993) and mRNA expression (Gupta et al., 1998), and by increased activity of protein kinase A (PKA; Gupta and Abou-Donia, 2001) and c-fos mRNA (Gupta et al., 2000b) in brain and spinal cord of hens treated with TOCP or *O,O*-diisopropyl phosphorofluoridate (DFP). Activated CaMKII produces hyperphosphorylation of cytoskeletal proteins: MAP-2 (Patton et al., 1983, 1985, 1986), tau (Gupta and Abou-Donia, 1999), α- and β-tubulin (Gupta and Abou-Donia, 1994; Suwita et al., 1986a, 1986b), neurofilament triplet proteins (Gupta and Abou-Donia, 1995a; Gupta et al., 1999), and myelin basic protein (Abou-Donia, 1995). This CaMKII-mediated aberrant phosphorylation of cytoskeletal proteins fits all of the criteria for OPIDN such as test compound specificity, dose dependence, time course of clinical condition, species specificity, and age sensitivity (Abou-Donia and Lapadula, 1990). Aberrant hyperphosphorylation leads to alterations in these proteins that are pathognomic representation of OPIDN. Elevated phosphorylation of MAP-2 reduces its ability to induce tubulin polymerization to form microtubules (Hoshi et al., 1988) and promotes disassembly of microtubules (Burns et al., 1984). Increased phosphorylation of tau diminishes the ability to bind to microtubules and results in destabilization with subsequent axonal degeneration (Gupta and Abou-Donia, 1999). Enhanced phosphorylation of tubulin prevents its binding to MAP-2 or its polymerization to microtubules (Wandosell et al., 1986) and induces their aggregation into twisted polymers distinct from microtubules (DeLorenzo et al., 1982). Hence, chlorpyrifos-induced tubulin phosphorylation that did not dephosphorylate formed stable adducts (Jiang et al., 2010). Increased phosphorylation of neurofilaments prevents their assembly into filaments (Hisanaga and Hirokawa, 1990); however, these neurofilaments form aggregates (Jensen et al., 1992; Gupta et al., 2000a) and exhibit slow axonal transport (Gupta et al., 1997). This abnormal axonal transport is also consistent with inhibition of caplain activity in hen sciatic nerve (Gupta and Abou-Donia, 1995a), leading to a decrease of neurofilament proteins in spinal cord of hens after treatment with DFP (Gupta and Abou-Donia, 1995b). These sequences of events lead to axonal degeneration and subsequent demyelination.

The results of this descriptive study support an association between neurologic deficits and levels of autoantibodies against neuron- and glia-specific proteins circulating in sera from a small cross-sectional sample of affected flight crew personnel compared to a small group of healthy controls. The results of testing four samples from a single pilot at different time points suggest a temporal association between exposure and biological damage. Data suggest that, although not diagnostic for a specific illness, the presence of circulating autoantibodies against neuronal and glial proteins may serve as further confirmation of chemical-induced nervous system injury in the absence of other neurologic diseases. These autoantibodies also may be used to monitor the progression of injury and recovery. Since autoantibodies identify damage to specific cells, these changes may help to identify cellular mechanisms underlying neurotoxicity.

It needs to be emphasized that although this study has presented intriguing findings, it

has its limitations. The sample size in this study was too small to examine important covariates like age and exposure. Another limitation in this study is the lack of availability of the identity of the chemicals and the levels to which the flight crew members were exposed. Development of biomarkers will help assess exposure, and also may help determine individual differences which may affect whether or not clinical illnesses develop (Schoper et al., 2010; Liyasova et al., 2011; Marisillach et al., 2011).

Although the findings of the study have certain limitations, it is clear that certain risks may be associated with exposure to cabin air emissions. Certain groups, including infants, the elderly, and the chronically ill, that are especially susceptible to toxic exposure to pyrolyzed engine oil and hydraulic fluid need to be protected by either operating planes with a nonbleed ventilation system or filtering the engine bleed air before it gets to the cabin and cockpit.

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