Autoantibody markers of neural degeneration are associated with post-mortem histopathological alterations of a neurologically injured pilot

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There are numerous concerns regarding the neurotoxicity of contaminated air inside pressurized aircraft. Neurological symptoms have been seen in many aircrew members who have reportedly been exposed to the breathable, yet potentially toxic, air in airliners. Symptoms allegedly contracted by aircrew and passengers are thought to be caused by a single large exposure or repetitive cumulative low-level exposures to toxic chemicals in the airliner internal air. Genetic variation plays a rôle. We report here the case of a 43-year old airline pilot who presented with neurological deficits and other symptoms. The pilot died without regaining good health. *In vivo* blood had been collected *ante mortem*. Analysis of the serum confirmed grossly elevated levels of serum autoantibody biomarkers for neuronal cell degeneration compared with a control group. At autopsy, various tissues underwent histopathological assessment. Brain and spinal tissues exhibited axonal degeneration and demyelination. Peripheral nerves showed T-lymphocyte infiltration and demyelination. T-lymphocytes had infiltrated the heart muscle tissue. The post-mortem tests and pathological examination of the nervous system confirmed the autoantibody biomarker results. Differential diagnosis showed that the work environment, clinical condition, histopathology and serum biomarkers for nervous system injury are consistent with organophosphate-induced neurotoxicity. The results also indicated that the inferred exposure to organophosphates sensitized the nervous system and heart tissue towards further injury.

Keywords: neural degeneration, lymphocytic myocarditis, pain

1. INTRODUCTION

This report presents the case of a 43-year old man ordinarily resident in the United Kingdom, a nonsmoker and abstinent, who complained of chronic ill health, and died in the Netherlands without regaining vigour. Just before death the subject attributed his symptoms to repeated exposure to engine oil fumes during the course of his employment as a commercial airline pilot. We present the results of routine medical evaluations and specialized tests, autopsy results and the levels of serum biomarkers for brain injury. The results of these tests are correlated with his ante-mortem clinical condition, and those of post-mortem examination of brain tissues. As far as the authors are aware, this is the first case study of a pilot presenting with chronic ill health following exposure to contaminated air that includes autopsy findings, comprising, *inter alia,* the histopathological examination of brain tissue.

The internal breathing air of all airliners (with the exception of the relatively new Boeing 787) is drawn in from the outside by the aircraft's main engines or auxiliary power unit (APU), using the compressor sections of these gas turbine engines. This "bleed air" is also used to heat and pressurize the cabin at high altitude and to pressurize the potable water tank, as well as the hydraulic system. It is suspected that the engine oil seals leak in daily use, and sometimes fail completely, allowing heated oil mist to escape into the bleed air [1–8]. The only air that enters such aircraft during operation is this "bleed air." Inadequate or improper maintenance practices, including overfilling the engine oil reservoir and failing to renew a worn or defective oil seal, a defective APU, or a failed bearing can each individually, or in combination, result in emissions (gaseous, vapour, and particulate constituents of pyrolysed engine oil and hydraulic fluid) [2] that contaminate the air-conditioning ducting [1] and are passed through to the cabin and flight deck [7, 8]. The engine lubricating oil contains tricresyl phosphate (TCP) (2–6% by weight), of which the tri-*ortho*-cresyl phosphate (TOCP) content is supposed to be less than 0.1% of the total TCPs, albeit that in reality the proportion might be much greater [9]. The oil also contains N-phenyl-1-naphthylamine, alkylated diphenylamines and phenyl dimethyl phosphate [10]. Hydraulic fluid contains tributyl phosphate (TBP), dibutyl phenyl phosphate (DPP), butyl diphenyl phosphate (BDP) or a mixture of all three [11, 12].

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It has been more than a decade since some pilots and flight attendants started to complain of nervous system-related symptoms following suspected exposure to air emissions inside aircraft [13]. The symptoms were hypothesized to have resulted from exposure to the organophosphates present in engine oil and hydraulic fluid [13]. There are three neurotoxic actions of organophosphates: first, cholinergic neurotoxicity caused by inhibition of acetylcholinesterase, followed by overstimulation of muscarinic and nicotinic acetylcholine receptors, with subsequent development of cholinergic toxicity [14]. Second, organophosphorus ester-induced delayed neurotoxicity (OPIDN), which is a central–peripheral axonopathy characterized by primary Wallerian-type axonal degeneration of the central (CNS) and peripheral (PNS) nervous systems, followed by secondary demyelination [15–18]; the clinical picture for OPIDN is manifested initially by mild sensory disturbances, ataxia, weakness and muscle fatigue and twitching, which may progress to paralysis. Third, organophosphorus ester-induced chronic neurotoxicity (OPICN) is characterized by long-term neurological and neurobehavioral deficits accompanied by brain neuronal cell death [14].

2. EXPERIMENTAL

2.1 Materials

For the tests carried out in the USA, NFP (bovine spinal cord), tau protein (human), MAP-2 (bovine serum), tubulin (bovine brain), and MBP (human brain), were from Sigma-Aldrich (St Louis, Missouri); GFAP (human) was from Biotrend Chemikalien (Cologne, Germany); and S100B (human brain) was from American Qualex International (San Clemente, California); horseradish peroxidase-conjugated goat anti-human IgG and enhanced chemiluminescence reagent were from Amersham Pharmacia Biotech (Piscataway, New Jersey); and SDS gels, 2–20% gradient (8 × 8), and tris-glycine 15 mM were from Invitrogen (Carlsbad, California). All other materials were purchased from Amersham.

2.2 Methods

2.2.1 Histopathology

Some peripheral nerves and parts of the central nervous system (CNS) were removed. Histopathological investigation of the peripheral nerves was carried out in the Netherlands.

2.2.2 Blood tests

A few months before the subject's death, blood had been drawn and serum prepared and stored at –70 °C. After death it was tested for circulating autoantibodies specific to seven proteins associated with the nervous system. Under a protocol approved by the Institutional Review Board at Duke University Medical Center, the results were compared with those of age-matched male controls.

Using a Western blot assay, all proteins were loaded at 10 ng/lane except for albumin, which was loaded at 100 ng/lane, using one gel for each serum sample, in triplicate [19]. Proteins were denatured and electrophoresed in SDS-PAGE (4 to 20% gradient). The proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Amersham). Nonspecific binding sites were blocked with tris-buffered saline–Tween (TBST) (40 mM tris, pH 7.6, 300 mM NaCl and 0.1% Tween-20) containing 5% nonfat dry milk for 1 h at 22 °C. Membranes were then incubated with serum samples at 1:100 dilutions in TBST with 3% nonfat dry milk overnight at 4 °C. After five washes in TBST, the membranes were incubated in a 1:2000 dilution of horseradish peroxidase-conjugated goat anti-human IgG. The membranes were developed by enhanced chemiluminescence using the manufacturer's protocol and signal intensity quantified using a Typhoon 8600 variable mode imager and Bio-Rad image analysis software (Hercules, California). All tests were investigator-masked.

Levels of autoantibodies against neural proteins were obtained by dividing the optical density (in arbitrary units) for subjects and controls by serum albumin optical density; the values for each subject were normalized to healthy controls and expressed as fold change from the controls [19].

Grouped data are reported as mean ± SE. The values from subjects were compared to the control group using a paired *t*-test. Mean values from the subjects' group were compared within groups using two-way ANOVA (SigmaStat; Systat Software). A P value < 0.05 was accepted as statistically significant [19].

3. THE SUBJECT

An outline of the subject's relevant life-events during his career is presented in Table 1. He began his flying career as an airline pilot in 1996. His career lasted for 15 years during which he flew a total of about 8000 hours.[1] Although he never reported a documentable fume emission, he said that he noticed that the engines, on start-up, would create puffs of smoke inside the aircraft (BAe ATP) accompanied by an oily smell. After three years of flying, in 1999, he started feeling that his brain was slow and he had some signs of confusion. In the year 2000 he

[1] He was also an enthusiastic, and extremely good, amateur paraglider, winning many competitions.

changed over to the Embraer 145 Jet. In 2006, while driving home after a flight, he had scintillating vision in one eye, moving from centre to side of the eye, and thereafter experienced three days of mental confusion. In 2007, the company he worked for was taken over by a major UK airline and he carried on flying for them, only now as an Airbus 319/320/321 line pilot. On 17 August 2011, while driving his car, he abruptly stopped while approaching a road junction, without apparent reason, and his car was run into by the following car, albeit at low speed.

Table 1. Timeline of events related to the subject's career.

№	Date	Events	Remarks
1	1996	Starting flying career: BAe ATP (advanced turboprop).	He was healthy and fit to fly
2	1996–1999	No documented fume event; when starting engines, they would create puffs of smoke inside the aircraft (BAe ATP); experienced oily smells.	No Symptoms
3	1999	First started feeling that his brain was slower than normal; some early signs of confusion began (after three years of flying).	Cholinergic
4	2000	Changed to Embraer 145 Jet.	
5	2006	While driving home after a flight, had scintillating vision in one eye moving from centre to side of the eye; three days of mental confusion.	Cholinergic
6	2007	Went on to fly Airbus 319/320/321 as a line pilot for a major UK airline.	
7	2008	Slow symmetric onset of numbness in hands and feet, creeping up as far as elbows and knees.	OPIDN
8	22 August 2011	Braked suddenly at a T-junction for no apparent reason. The following car collided with his. Whiplash was suspected. Prescribed Naproxen 500.	
9	22 August 2011	Continued to fly for his airline.	OPIDN
10	2 September 2011	Last flight.	
11	8 September 2011	No improvement in his condition and not sleeping at night; Zopiclone 7.5 mg before bedtime. Blood was taken with no remarkable results. An MRI was prescribed. Paresthesia in both legs and both hands.	Cholinergic/OPIDN
12	17 September 2011	Symptoms became worse. He arranged his own CT and MRI scans; Went to see an osteopath without referral. Went to the A&E (ER) because of severe tightness and pain in his chest. Continued to have difficulty walking; had ataxia.	OPIDN
13	25 September 2011	Attended again at the ER as he felt "uncoördinated".	OPIDN/OPICN
14	23 January 2012	A psychiatrist referred him to a psychiatric hospital. In-patient for one month.	
15	23 February 2012	Discharged himself from the psychiatric hospital. Thereafter he requested and obtained a subcutaneous fat biopsy: organophosphate metabolites (6 months after his last flight). Test revealed low ATP level.	
16	5 April 2012	Consultation in the Netherlands: Insecure staggering and heavy gait, walking difficulty, signs of being in severe and constant pain and desperate, pain in moving eyes, headache, tremors, some neck stiffness, slow mental processes, sharp decline in memory. Accepted in an out-patient clinic.	OPIDN/OPICN
17	23 July 2012	Amsterdam: MRI. No structural deficits were found to explain his loss of functions.	
18	27 September 2012	Amsterdam: Neurologist; Still suffering from serious neurological complaints. Serious doubt that he will ever fly again in the foreseeable future.	
19	19 October 2012	Amsterdam: Extensive neuropsychological tests; substandard performance. Memory was poor and he seemed to be always trying to mask the effort to hide his deficits.	OPICN
20	6 December 2012	Amsterdam: fMRI; no diagnosed abnormalities.	OPICN
21	5 May–11 December 2012	Amsterdam: Regular QEEG; daily neurotherapy provided substantial relief of complaints in his head. He was lucid, very puzzled, and inquisitive. He had been losing weight. No one was aware that he was taking pentobarbital or when he had started. He was waiting for an appointment at the pain clinic at Amsterdam University Hospital.	OPICN
22	12 December 2012	Found dead in hotel room. He was a 43-year old airline pilot on sick leave and was still on full pay.	

His last flight was on 2 September 2011. On 17 September 2011 he went to the Accident & Emergency unit (A&E) (USA: ER) because he felt severe pain and tightness in his chest. Standard A&E cardiological tests did not reveal any cause; he was kept in overnight and discharged the next morning with no diagnosis. A week later he went back to A&E as he "felt uncoordinated" and said all his symptoms had become worse. A&E discharged him, again with no diagnosis, and scheduled (nonurgent) scans. Upon examination on 22 September 2011, his general practitioner (family doctor) noted "Numbness or pain in all limbs. Headaches in occipital area. Finds walking and co-ordination difficult and veers to the right. Brain doesn't know what legs are doing." An appointment was made for him to see a neurosurgeon after a week and he was prescribed Zopiclone 7.5 mg, one dose at bedtime. The next day he was prescribed Amitriptylene 10 mg, 1 or 2 doses at night. This was later stepped up to 3 to 4 doses at night as he couldn't get to sleep because of pain. The medical notes record instances of sleep-apnoea, and he was referred to a sleep clinic. Being of athletic build (185 cm/70 kg) and bearing no excess fat, the subject was therefore not a typical sleep-apnoea patient. The neurosurgeon subsequently reported that no surgical intervention was appropriate.

On 17 November 2011 the subject was referred for evaluation to a consultant psychiatrist, whom he saw several times and, in January 2012, he was admitted to a psychiatric hospital. The subject felt that because "his doctors couldn't ascribe a diagnosis at all, they may as well evaluate his mental condition". He remained an in-patient there for four weeks and then discharged himself. There are no recorded psychiatric symptoms in his medical notes. He was prescribed antidepressants and benzodiazepines. He had undergone many tests, including of his blood, in the UK, but all the results were negative and did not offer any diagnosis.

On 23 February 2012, just after he discharged himself from the psychiatric hospital, a test (for which he self-referred himself) showed low levels of intracellular adenosine triphosphate (ATP) and a subcutaneous fat biopsy showed organophosphate (OP) metabolites. This was approximately six months after his last flight.

All in all, he was examined by no less than 15 specialists, including neurologists, while still resident in the UK. In spite of this there was no diagnosis of his ill health.

On 5 April 2012, he showed up, unannounced, in an Amsterdam clinic still having an insecure, staggering and heavy gait, walking with difficulty and with neck stiffness. His mood was one of desperation; he also experienced severe fatigue with constant pain; he suffered severe headaches, slow mental processes, a sharp decline in memory, and pain when moving his eyes. He continued to have difficulty walking. He had ataxia. He would fall off his bicycle for no reason. He remained in the Netherlands until he died some 9 months later.

MRI scans on 23 July 2012 and 6 December 2012 showed no structural defects to explain the loss of function in the patient. He also had an fMRI scan, but no abnormalities were found to explain his symptoms. On 27 September 2012 he was still suffering from serious neurological complaints. On that date his neurologist expressed serious doubts whether he would ever be well enough to fly in the foreseeable future. On 19 October 2012 his clinical psychologist gave him extensive neuropsychological tests and concluded that his performance was substandard. From 5 May to 11 December 2012, at his own request, at the Center for Neurotherapy (NCH Hilversum) regular quantitative electroencephalography (QEEG) was carried out daily and he received neurotherapy, which provided substantial relief of the complaints in his head.

On 12 December 2012 the subject was found dead in his hotel room, as a 43-year old airline pilot on sick leave and still on full pay. His condition prior to his sudden death was essentially lucid, puzzled and inquisitive. He had lost weight. On occasions he was in extreme pain, mainly in the head; he was waiting for an appointment at the pain clinic at Amsterdam University Hospital. He had acquired a "sleep-angel" (an electronic device that sounds an alarm when breathing is not detected for a preset interval). This had been "armed" by him as usual before he went to bed on 11 December. When hotel staff entered his room, the alarm was still sounding. In bed, he wore a mouth-guard, the effect of which was to keep the lower jaw proud of the upper jaw during sleep. When he was found dead in his bed he was still wearing it. (These two devices are often recommended for persons suffering from sleep-apnoea.)

4. DIAGNOSIS

As mentioned, while in the UK the subject was, remarkably, never diagnosed, despite his chronic illness, constant pain and a long list of complaints from 1999 to 2012, including almost constant severe headaches, occasional severe chest pain, short-term memory loss, confused mental processes, cognitive dysfunction, apnoea, numbness that he described as "pins and needles", clumsiness, a tendency to fall easily, and fatigue; he was seen by many specialists, underwent several tests and was admitted more than once to hospital. Shortly before he died in the Netherlands, he was diagnosed as suffering from the consequences of exposure to organophosphates. The

primary basis of this diagnosis was that his symptoms were consistent with those known to be caused by exposure to the organophosphates present in jet engine oil and hydraulic fluid. The first sign of neurological deficits consistent with OPIDN had been in 2008, when he experienced a slow onset of numbness in his hands and feet, creeping up as far as the elbows and knees, respectively. It is unknown whether he went to a medical doctor with these complaints. They are typical of the earliest manifestations of OPIDN. In August 2011 he had paresthesia in both legs and both arms, which is a hallmark of OPIDN. The application of differential diagnosis techniques, together with the results of the extensive consultations and tests already carried out in the Netherlands and in the UK, offered no plausible alternative diagnosis.

5. POST-MORTEM REPORTS

Due to the apparently unnatural nature of this death, two autopsies were carried out, one for the local police and another for the subject's family, the latter by one of the present authors (FRWvdG).

5.1 Brain weight

The subject's brain weighed 22% more than an average healthy adult brain [20]; this indicated fluid accumulation in the brain.

5.2 Toxicology report—pentobarbital levels

Toxicology tests on the blood revealed the presence of pentobarbital (also known as pentobarbitone) at a potentially lethal level [20]. The Netherlands Forensic Institute (NFI), where the subject's first autopsy took place, reported that the level of pentobarbital in the femoral blood was 27 mg/L, and its concentration in heart blood was 32 mg/L. Pentobarbital concentration in eye fluid was 14 mg/L. The second, family-instructed, autopsy reported the femoral blood level at 22.3 mg/L (average for femoral blood: 24.65 mg/L) and in the hair pentobarbital was detected at 3.84 ng/μg. Hair testing indicated that he had been taking this medication during the preceding months at therapeutic doses. The brain was not tested for pentobarbital.

Pentobarbital is generally a prescription-only medicine. It is a short-acting (half-life is 21–42 hours) sedative-hypnotic barbiturate. At therapeutic doses, barbiturates reversibly depress activity of all excitable tissues, with the central nervous system (CNS) being the most sensitive, and there is very little effect on skeletal, cardiac or smooth muscle. Only in acute intoxication is depression extended and serious deficits in cardiovascular and other peripheral functions occur. Oxygen consumption in various tissues, and respiration in the mitochondria, can be depressed by barbiturates at high concentration [21].

There was no evidence that the subject had been prescribed this medication and those treating him were unaware that he was apparently taking it [20]. The police did not find any medicine container for this drug among his possessions. This raises two reasonable presumptions: (i) there was nothing to indicate to him a safe dose; and (ii) he may not even have been aware that the substance was pentobarbital. Note that the drug can be purchased on the black market or via the internet. It is thought that he was taking it because he had difficulty going to sleep or to relieve excessive pain.

5.3 Summary of autopsy findings

In brief, the two autopsy reports indicated the following (there were no material discrepancies between the two):

1. The subject's death "can well be explained by functional disorders of the brain and the heart, on the basis of tissue damage in both these organs."
2. "The pentobarbital found in the blood of the subject at the levels observed contributed to death by its depressant effects on the central nervous system. On the basis of the levels observed in the toxicological examination performed, the death of the subject can be attributed to pentobarbital."
3. The autopsy yielded indications of a recent oxygen deficiency in the heart and brain, which caused damage to the cardiac muscle with signs of herniation of the brain. "As such, this person's death can well be explained by functional disorders of the heart and brain."
4. The autopsy cardiopathological examination revealed pathological abnormalities in the heart; i.e., inflammation of the cardiac muscle and narrowing of the coronary arteries. These abnormalities may have resulted in cardiac dysfunction leading to damage to the cardiac muscle, which may subsequently have led to an oxygen deficit in the brain, resulting in brain herniation and death.
5. Two causes can be identified that, either independently or in combination, may have led to the oxygen deficiency resulting in the subject's death:
   a. In the toxicological examination, pentobarbital was found in the body. The toxicologists concluded that the measured concentrations of pentobarbital can explain the subject's death. Due to its effect as a central nervous system depressant at the concentrations established, pentobarbital may have caused an imbalance between oxygen supply to and removal from the brain and the heart, resulting in a lack of oxygen, tissue damage and damage to the cardiac muscle, herniation of

the brain, organ dysfunctions and death. The finding of fluid in the lungs is unspecific but may be consistent with the toxic effects of pentobarbital.

b. The autopsies and supplementary cardiopathological examinations revealed pathological abnormalities in the heart; i.e., inflammation of the cardiac muscle and narrowing of the coronary arteries, which may have resulted, separately or in combination, in cardiac dysfunction leading to damage of the cardiac muscle.

6. HISTOPATHOLOGICAL ASSESSMENT

6.1 Heart tissues

Examination of the heart tissues revealed myocardial autolysis and an excess of lymphocytes in many places in the myocardium, mainly loose between muscle fibres but also grouped and present particularly in the epicardium. Myocytolysis was noted. Striking was the presence of a relatively high amount of lymphocytes in the myocardium. There was thickening of the arterial walls with lymphocytic infiltrate. Both pathologists therefore reported lymphocytic myocarditis. Fig. 1 demonstrates lymphocytic infiltration in the heart muscle.

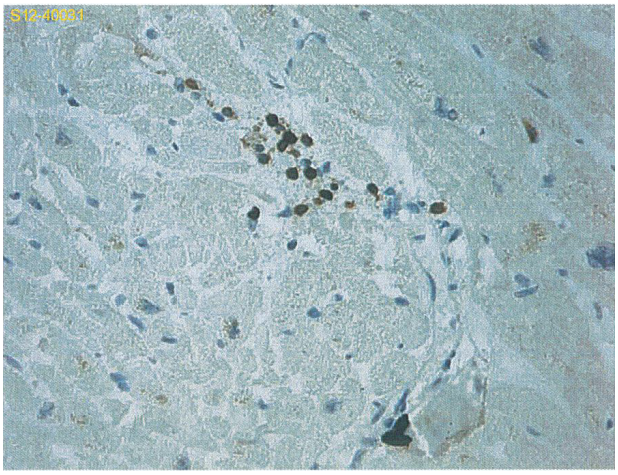

Figure 1. Heart muscle infiltrate of T-lymphocytes. Micrograph width = 100 μm, 4×.

The mediastinum, including the thorax and the associated fatty tissues, displayed an excessive number of lymphocytes. The diaphragm appeared normal, but in the adjacent connective tissue, blood vessels and nerve branches there was extensive lymphocytic activity, with a large number of T-lymphocytes and a diminished number of B-cells.

6.2 Sciatic and femoral nerves

These nerves showed an endoneural T-lymphocyte invasion. A pathological determination of peripheral endoneuritis was made. In addition, the peripheral nerves showed gross axonal demyelination. This is demonstrated in Figs 2 and 3.

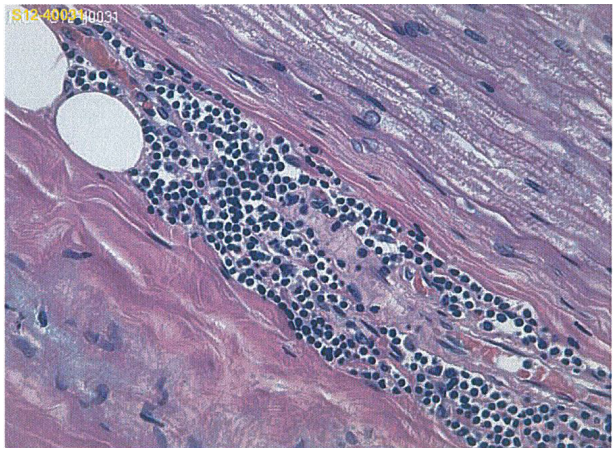

Figure 2. Demyelination (white material largely absent) and lymphocytic invasion (black dots) of peripheral nerve. Micrograph width = 100 μm, 4×.

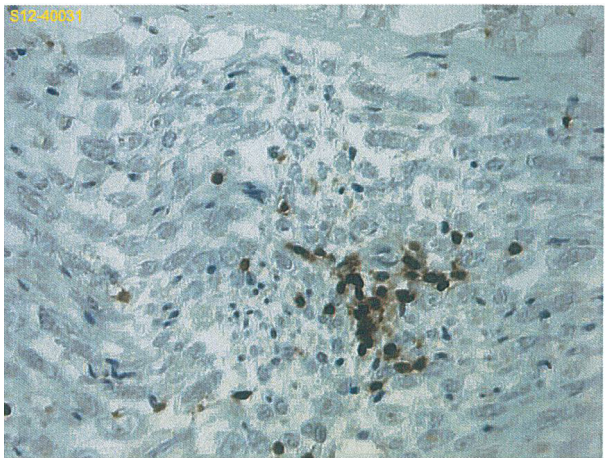

Figure 3. T-lymphocyte infiltration of peripheral nerve. Micrograph width = 100 μm, 4×.

Absence of axonal degeneration in peripheral nerves is consistent with the results of OPIDN in laboratory animals. Experimental studies of OPIDN have shown absence of peripheral nerve pathological changes in animals long after developing OPIDN. This is consistent with the regenerative capacity of the peripheral nervous system. Previous studies have indicated that, although damage to both the CNS and PNS may contribute to neurological dysfunction in OPIDN, CNS lesions are more significant because they are irreversible, whereas the PNS can regenerate. This conclusion is consistent with the spasticity seen in human patients exposed to TOCP and the insecticide leptophos, which are capable of causing OPIDN, suggesting injury of the CNS [22]. In experimental studies, ataxia correlates with consistent early occurrence of spinocerebellar tract degeneration in the posterior and lateral columns of affected cats [23].

6.3 The central nervous system

Various sections of the CNS (brain, brain stem and spinal cord) were subjected to histological examination. Samples were drawn from the frontal cortex, hippocampus, cerebellum and spinal cord. The tissues had been fixed in formalin. They were dehydrated and embedded in paraffin wax. Sections were stained with haematoxylin–eosin (H&E) alone or in combination with Luxol fast blue (LFB). The test looked for neuronal cell death and demyelination. The H&E stains the tissues pink and dead cells are remarkable as dark-stained matter. After staining the material with H&E, the LFB was used to stain the myelin blue. Where demyelination had occurred, areas of myelin that should then have been blue are seen as areas of pink.

6.3.1 Cortex

The frontal cortex exhibited clear neuronal cell loss and prominent spongiosis. The prefrontal cortex showed increased glial cells and macrophages. Spongiosis was also present here, as well as shrunk and dying neuronal cells (Figs 4–7). This material also demonstrated demyelination under low and high magnification.

Demyelination was noteworthy in the cortex sections. This is demonstrated in Figs 8 and 9.

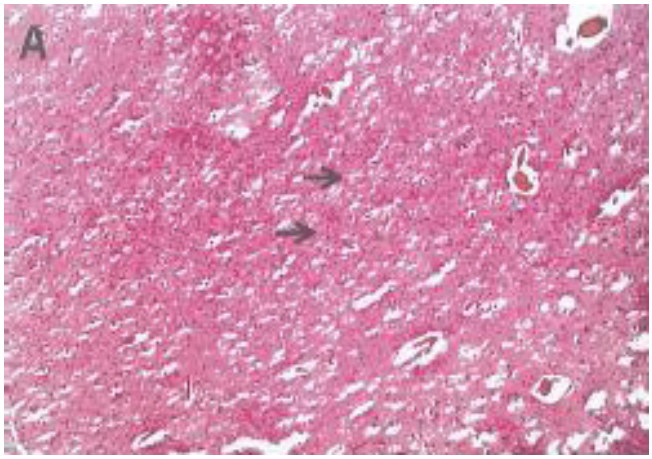

Figure 4. Section of frontal cortex at low magnification showing neuronal cell loss and prominent spongiosis (arrows). Micrograph width = 100 μm, 4×.

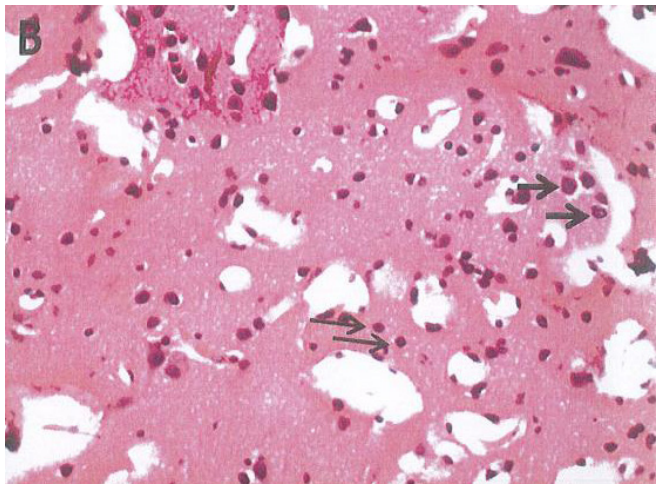

Figure 5. Sections through prefrontal cortex at intermediate magnification noting increased glial cells (longer arrows) and macrophages (shorter arrows). Micrograph width = 100 μm, 20×.

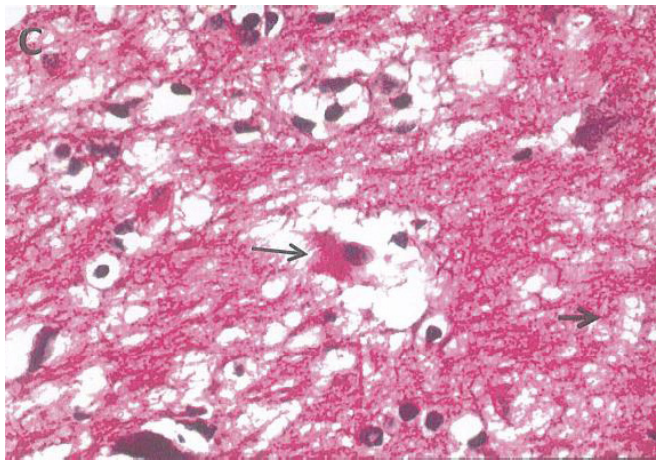

Figure 6. Highly magnified sections through the prefrontal cortex showing dying neuronal cell indicated by the longer arrow. Spongiosis is indicated by the shorter arrow. Micrograph width = 100 μm, 40×.

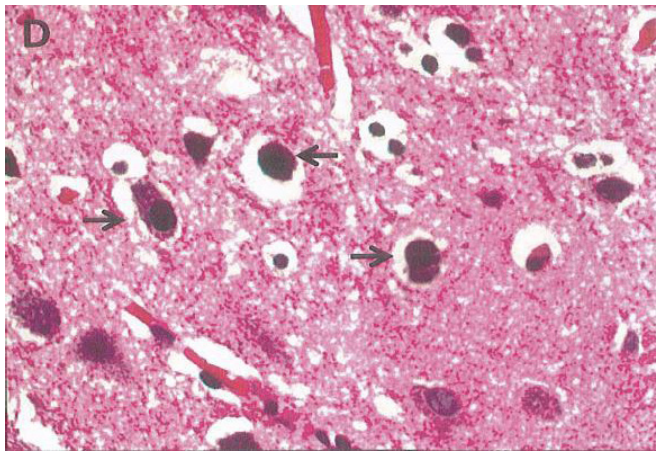

Figure 7. Highly magnified section through the prefrontal cortex showing shrunk and dying neuronal cells indicated by arrows. Note the dense chromatin in the dying cells. Micrograph width = 100 μm, 40×.

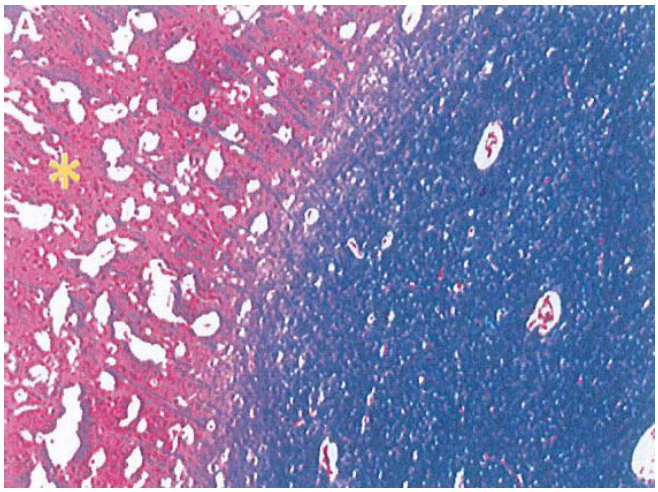

Figure 8. Low magnification section of cortex stained with H&E and LFB (colour online) with yellow asterisk indicating area of demyelination. Micrograph width = 100 μm, 4×.

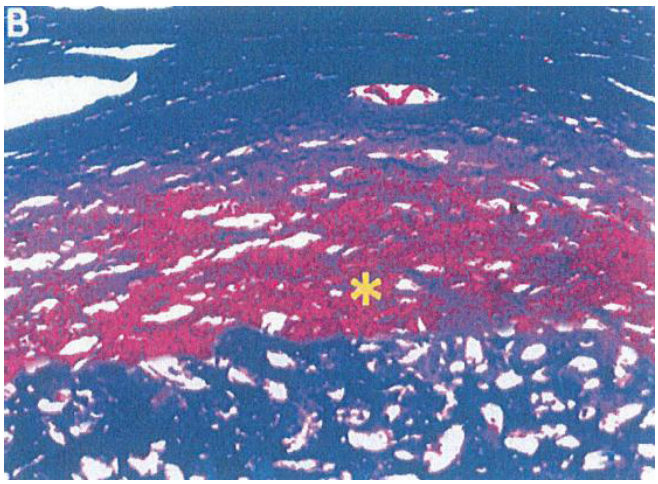

Figure 9. Intermediate magnification section of cortex stained with H&E and LFB (colour online) with yellow asterisk indicating area of demyelination. Micrograph width = 100 μm, 20×.

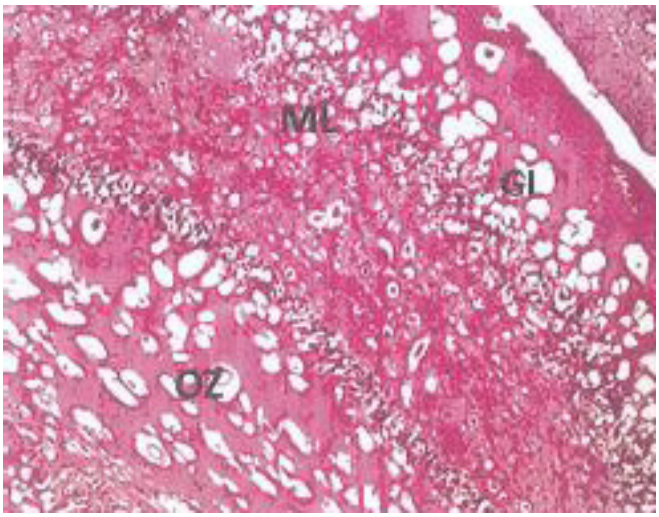

Figure 10. Section of dentate gyrus showing spongiosis in the outer zone (OZ) and in the molecular (ML) and granular (GL) layers. Micrograph width = 100 μm, 10×.

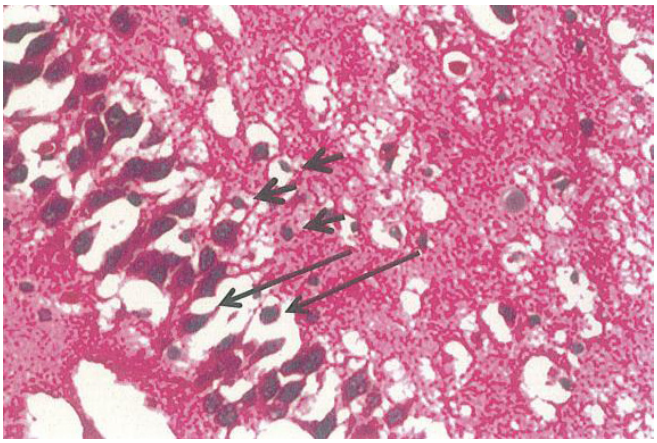

Figure 11. Section of dentate gyrus at high magnification showing apoptotic cells with dense chromatin nuclei (longer arrows) and astrocytes (shorter arrows). Micrograph width = 100 μm, 40×.

6.3.2 Hippocampus

The hippocampus tissue was stained using the same stains and the same technique. The dentate gyrus showed slight spongiosis in the outer zone and in the molecular layer. With higher magnification there was clear evidence of apoptotic cells and astrocytes. These are demonstrated at Figs 10 and 11.

The hippocampus also had demyelinated cells. This is demonstrated in Figs 12 and 13.

6.3.3 Cerebellum

Stained and examined in the same way, the cerebellum showed substantial cell loss in the Purkinje cell layer, the molecular layer and the granular layer. Clear evidence was observed of a degenerated, damaged and shrunken Purkinje cell layer. The damaged Purkinje cells were hyperchromatic. The damage to the Purkinje layer is depicted in Figs 14–16.

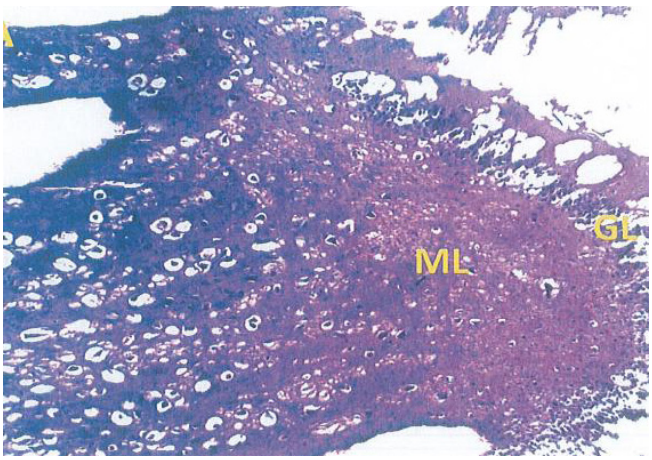

Figure 12. Section of hippocampus outer layer and molecular layer (ML) at low magnification showing demyelination where the blue staining is missing and allows the pink staining (colour online) to remain. Micrograph width = 100 μm, 4×.

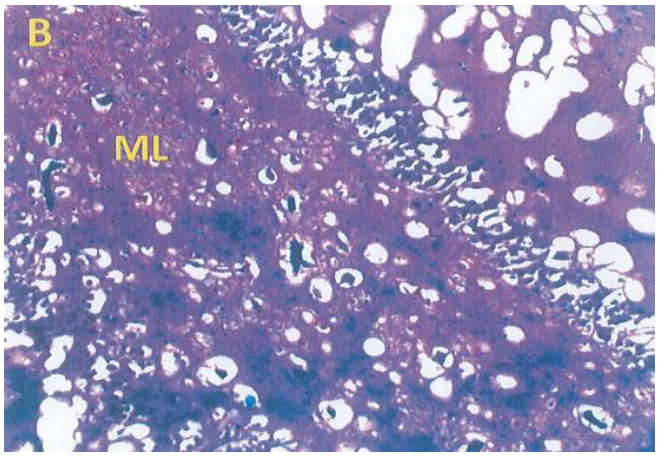

Figure 13. Section of hippocampus dentate gyrus showing areas of demyelination. The blue staining (colour online) gives way to pink where the myelin is missing. Micrograph width = 100 μm, 20×.

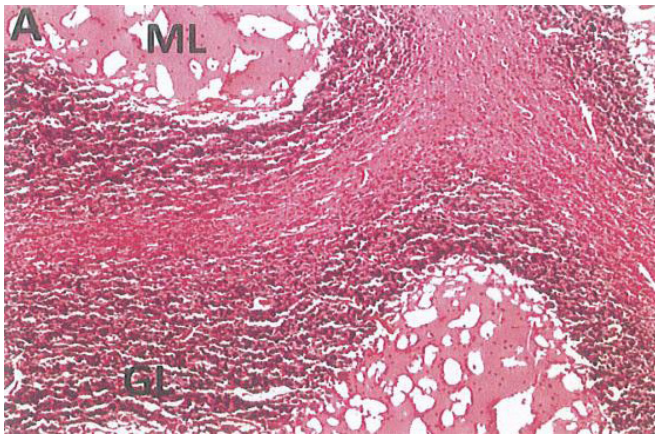

Figure 14. Low magnification section of cerebellum showing substantial cell loss in the Purkinje, molecular and granular layers. Micrograph width = 100 μm, 4×.

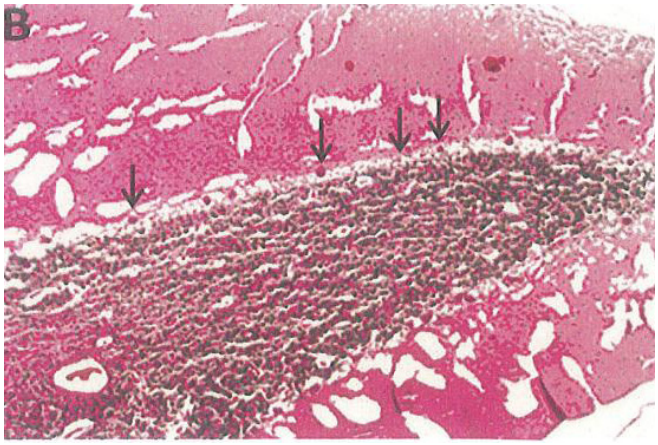

Figure 15. Section of the cerebellum showing substantial loss of Purkinje cells. Arrows indicate dead Purkinje cells. Micrograph width = 100 μm, 10×.

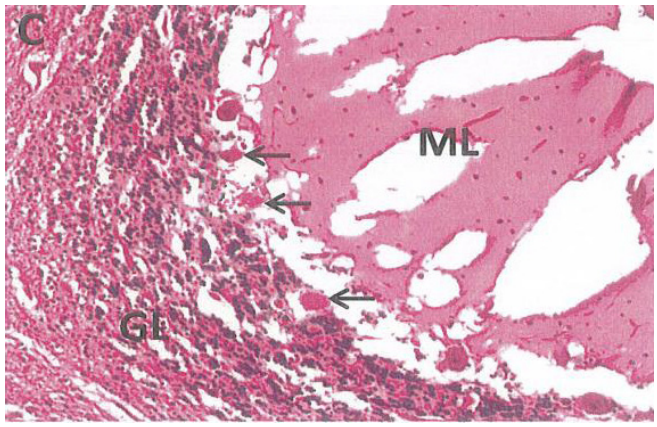

Figure 16. Degenerated Purkinje neurons in a pinkish colour (online) are denoted by arrows. Micrograph width = 100 μm, 20×.

Staining the cerebellum with H&E revealed Purkinje cells that are damaged, shrunken and with a hyperchromatic (darkly stained) basophilic perikaryon; they are indicated in the affected cells by the arrows in Fig. 16.

The cerebellum was also demyelinated, seen when stained with LFB (see Figs 17 and 18).

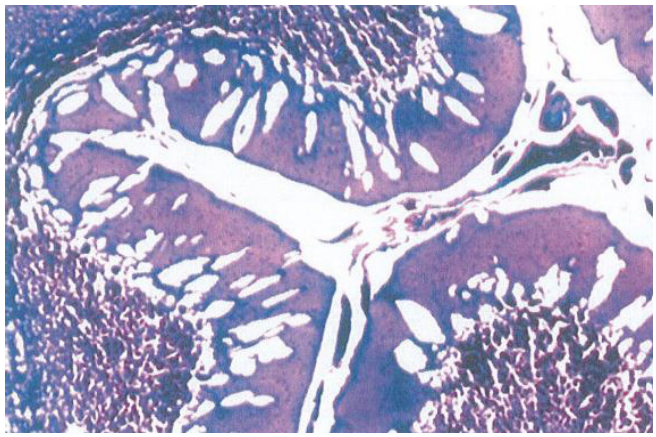

Figure 17. A low magnification section showing demyelinated cells, where blue gives way to pink (colour online). Micrograph width = 100 μm, 4×.

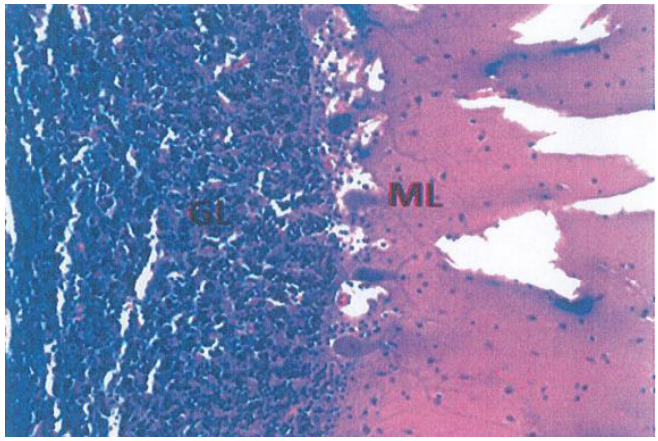

Figure 18. A section demonstrating slight demyelination of the granular layer (GL) and pronounced demyelination of the molecular layer (ML). Micrograph width = 100 μm, 20×.

6.3.4 Spinal cord

Areas examined were grey matter, white matter and the central canal. Significant findings included areas of grey matter that contained motor neurons with normal Nissl substance (Figs 19–21). This grey matter, however, contained macrophages and lymphocytes, and shrunken and dying hyperchromatic cells.

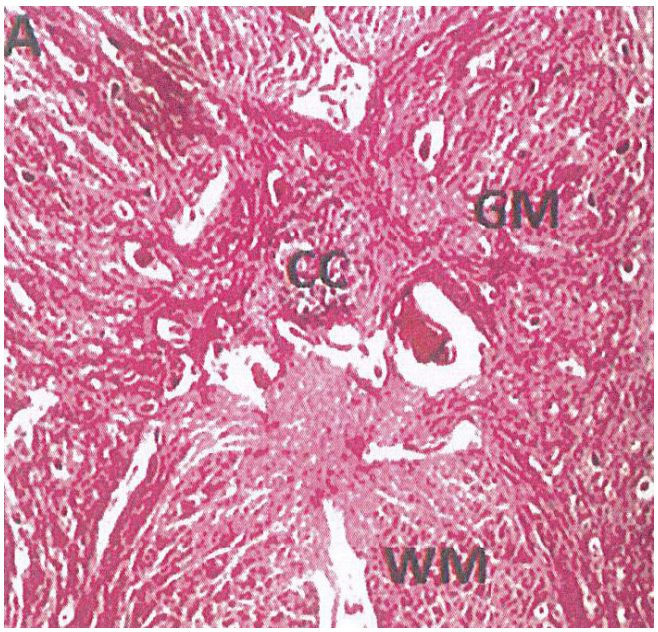

Figure 19. At low magnification, showing areas of white matter (WM), grey matter (GM) and the central canal (CC). Micrograph width = 100 μm, 4×.

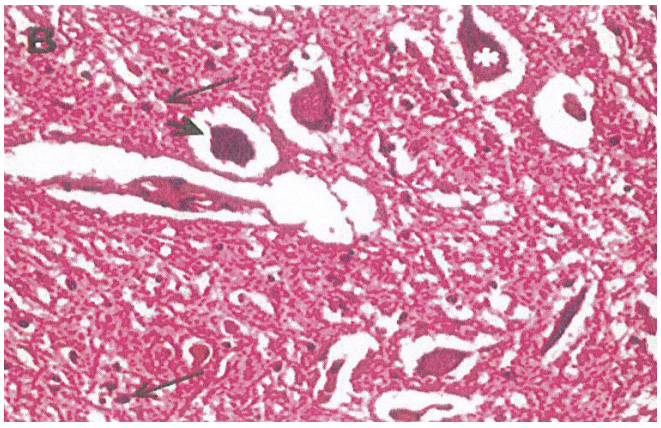

Figure 20. Highly magnified grey matter is seen to contain motor neurons (white asterisk), macrophages (longer arrows) with the presence of lymphocytes. An example of a shrunken and dying hyperchromatic cell is shown by the shorter arrow. Micrograph width = 100 μm, 40×.

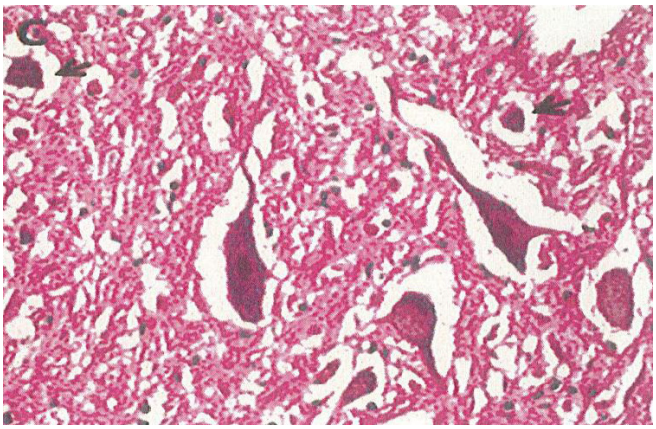

Figure 21. Grey matter showing shrunken and hyperchromatic dying cells (arrows). Micrograph width = 100 μm, 40×.

Areas of spinal cord showed clear evidence of demyelination when stained with LFB. This is demonstrated in Figs 22 and 23.

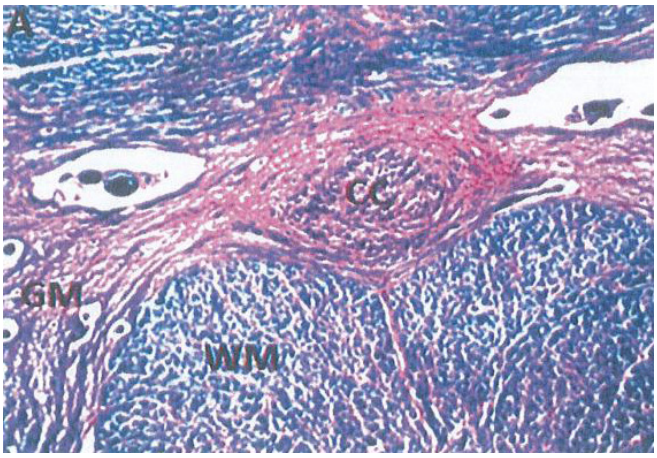

Figure 22. A section of spinal cord at low magnification shows demyelination of white matter (WM), grey matter (GM) and central canal (CC). Micrograph width = 100 μm, 4×.

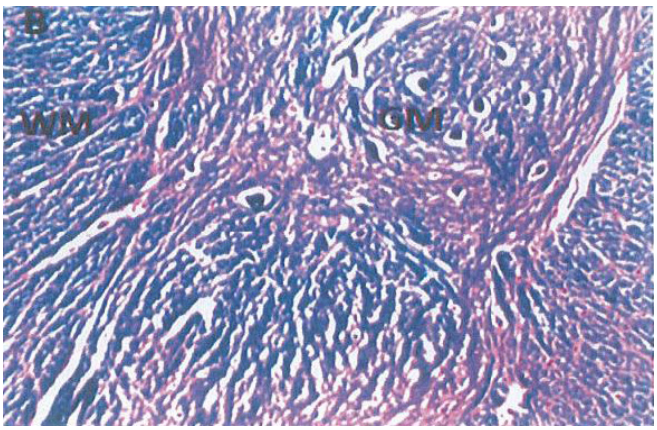

Figure 23. This slide at intermediate magnification shows demyelination more pronounced in the grey matter (GM) than in the white matter (WM). Micrograph width = 100 μm, 20×.

The above histology results are also confirmatory of the ante-mortem diagnosis of CNS deficits.

7. NEUROSPECIFIC AUTOANTIBODIES

In the serum of the subject we identified and quantified IgG autoantibodies against cytoskeletal proteins associated with neuronal and glial degeneration (Table 2). These were associated with: (1) neurogenesis; i.e., neurofilament triplet proteins (NFP), tubulin, microtubule-associated protein tau (tau protein) and microtubule-associated protein 2 (MAP-2); (2) myelinogenesis; i.e., myelin basic protein (MBP); and (3) astrogliogenesis; i.e., glial fibrillary acidic protein (GFAP) and S100B. Both GFAP and S100B are secreted by astrocytes.

Table 2. Levels[a] of serum autoantibodies (AA) in controls and the subject and subject's fold increases relative to healthy controls.

Brain-specific protein	Neurological function[b]	AA level		Subject level (fold of healthy control)[c]	Location of tissue injury[b]	Associated neurological deficits[b]
		Control mean ± SE	Subject mean ± SE			
Neurofilament protein (NFP)	Neurogenesis Axonal development and axonal transport	0.59 ± 0.17	7.20 ± 0.54	12.2 ± 3.6	Axonal degeneration	1. Cerebral cortex weakness, deficits in posture, locomotion; deficits in movement of fingers, speech and facial expression. 2. Limbic system learning, memory deficits
Tau protein (τ)		0.86 ± 0.25	3.97 ± 0.38	4.6 ± 1.4		
Tubulin	Axonal transport Present in other tissues	1.54 ± 0.23	9.44 ± 0.86	6.1 ± 1.1	Axonal degeneration and damage to other tissues	
Myelin basic protein (MBP)	Myelino-genesis Myelin development	0.75 ± 0.13	13.91 ± 1.10	18.5 ± 3.5	Demyelination	
Microtubule-associated protein-2 (MAP-2)	Neurogenesis Dendrite development of nerve cell	1.51 ± 0.13	21.61 ± 1.23	14.3 ± 1.5	Dendrite degeneration	Purkinje cells (cerebellum) Incoördination, staggering, ataxia
Glial fibrillary acidic protein (GFAP)	Gliogenesis Forms scar in injured axons	0.84 ± 0.25	8.58 ± 1.34	10.2 ± 3.4	Axonal injury	Chronic axonal injury, blockage
S-100B protein (S-100B)	From astrocytes in acute injury	0.25 ± 0.06	1.64 ± 0.12	6.6 ± 1.6	Acute, traumatic brain injury	Acute axonal injury

[a] The results are expressed as mean values of triplicate assays of optical density units normalized to albumin optical density.
[b] See the main text for fuller descriptions and references to sources.
[c] The values from the subject were compared to the control group using the paired *t*-test and were all highly significant ($P < 0.001$).

Table 2 lists the levels of autoantibodies against neural proteins for controls and the subject and the increase of the subject's autoantibodies relative to the healthy controls. The test shows that the pilot's autoantibodies were highly significantly elevated against nervous system-specific proteins compared with the controls. This finding is consistent with severe neuronal damage; that is, it confirms the ante-mortem diagnosis of severe neural damage.

8. DISCUSSION

This report presents the results of tests carried out on a pilot of commercial aircraft who flew for 15 years before his untimely death. Three years after he started flying, he began complaining of chronic ill health (which he attributed to breathing toxic substances in the flight deck air). A few months before his sudden death at age 43 he gave a sample of his blood to evaluate autoantibodies against specific proteins that are biomarkers for brain injury. During his 12 years of chronic illness he was examined by several physicians, admitted to hospital several times, and underwent many tests, but he was never diagnosed as suffering from any disease. Shortly before his death he went to the Netherlands, where he was diagnosed as suffering from organophosphate-induced neurotoxicity.

8.1 Autoantibodies against nervous system-specific proteins as biomarkers for brain injury

Nervous system injury results in neuronal degeneration, demyelination, and glial damage. Subsequently, nervous system-specific proteins are released into circulation. These proteins are short-lived because they ultimately reach the liver where they are degraded [19]. Proteins derived from damage to the nervous system act as antigens and react with plasma cells (derived from B-lymphocytes) to form autoantibodies. Initially, after a time lag of approximately four days, plasma cells produce small amounts of the short-lived IgM type, which accounts for approximately 10% of immunoglobulin.

Exposure of the memory plasma cells to the same antigen at a later time results in a secondary immune response, and it rapidly switches to produce greater quantities of IgG, IgA or IgE. IgG is the major circulating antibody, accounting for approximately 70% of immunoglobulin. The early appearance and long survival of autoantibodies to the nervous system-specific proteins permit practical surveillance of exposure and toxicity. Therefore neurological symptoms, along with IgG, IgM, and/or IgA autoantibodies against neurotypic- and glyotypic-specific proteins, are important in the pathogenesis and diagnosis of nervous system injury.

The autoantibody results show significantly increased autoantibodies against brain-specific cytoskeletal proteins, consistent with neuronal and glial degeneration. The levels of autoantibodies against nervous system-specific proteins were much higher (except for tau and tubulin) than the mean levels of the 34 cases of ill flight crew members previously reported [19]; the present case showed the following fold changes over the ones reported in ref. 19: NFP: 4.5; τ: 0.74; tubulin: 1.1; MBP: 4.4; MAP-2: 4.2; GFAP: 3.3; and S100B: 14.1. Cytoskeletal proteins are major targets of chemical-induced injury of the brain. Neurofilaments (NF) are major constituents of the axon, accounting for 80% of its protein [23–25]. NF provide rigidity and support; they are assembled from three subunits in a substoicheiometric ratio. The microtubule-associated protein τ is an axon-specific cytoskeletal protein that binds to and stabilizes microtubules [26]; besides maintaining neuronal architecture, τ plays a pivotal rôle in brain development and synaptic plasticity [27]. Loss of τ results in neurodegeneration and cognitive deficits [27]. MAP-2, the most abundant protein in the brain, is located in neuronal cell bodies and dendrites; it helps stabilize microtubules and mediate interactions with other organelles having microtubules [27]. Increased serum autoantibodies against MAP-2 are consistent with organophosphate-induced injury of neurons belonging to the cerebral cortex and the CA1 subfield of the hippocampus [28]; previous reports showed that degradation of MAP-2 following exposure to neurotoxic chemicals in the cerebral cortex and hippocampus is the result of global ischaemia [29]; abnormal phosphorylation of MAP-2 by organophosphate-induced activation of calcium calmodulin kinase II (CaMKII) may impair the normal structure and function of neurons [30]. Myelin is produced by oligodendrocytes (supporting cells located in the CNS); loss or damage of myelin is associated with demyelinating signalling and nervous system diseases such as multiple sclerosis (MS) and the release of myelin basic protein (MBP). GFAP plays an important rôle in the long-term maintenance of brain cytoarchitecture, proper functioning of the blood–brain barrier, and modulation of neuronal function [31]; the finding in this case of highly significantly increased autoantibodies against GFAP is consistent with axonal degeneration and with previous reports that individuals with neuropsychiatric disorders have elevated levels of GFAP [31, 32]. S-100B, a small calcium-binding protein produced mostly by astroglial cells of the central nervous system, exerts both detrimental and neurotrophic effects depending on its concentration in brain tissues; traumatic acute injury results in great destruction of astrocytes leading to massive release of S100B (up to fiftyfold) into plasma; its levels in psychiatric disorders have been found

to be higher in patients compared to controls, correlating well with its neuroprotective action [33].

It is realized that the autoantibody test detects only damage to the nervous system. It is also accepted that this test is unable to confirm whether the cause of the neural damage was neurotoxic contamination of the aircraft cabin air. Nevertheless, it is possible to form a view as to whether the results are consistent with damage observed time and time again in aircrew following known and documented exposure to organophosphorus compounds. It is also possible to correlate the results with the neurological deficits and symptoms reported by a surfeit of aircrew who are affected and grounded [4, 19, 34]. Aircrew are said to be at risk because they happen to fly frequently, not because they manipulate the controls or apparatus of an airliner (a corollary of which is that passengers, too, may be at risk if they fly frequently).

Autoantibodies against neurofilament proteins have been detected in the serum of some individuals who were exposed to arsenic and developed neurological deficits [35] and in a child who became quadriplegic after exposure to TCP [36]. Autoantibodies against NFP, GFAP and MBP were detected in the serum of a teenager who was exposed to the organophosphorus insecticide methamidophos [37]. An experimental study showed that autoantibodies to NFP, MBP, and GFAP were elevated in hens exposed to the active metabolite of TOCP (i.e., phenyl saligenin phosphate) and they developed OPIDN [36, 38].

8.2 Nervous system inflammation

In the central nervous system, only very few T-lymphocytes are found under healthy conditions. Accumulation of infiltrating T-cells can occur under inflammatory conditions, characterized by increased activation and proliferation of microglial cells. The T-cell infiltration occurs initially synchronously to the induced neurodegeneration and has more $CD4^+$ than $CD8^+$ T-cells, suggesting complementary rôles in the disease [39]. Dying cells and/or the accumulation of debris or aggregated proteins can occur in the CNS, e.g. as a result of exposure to neurotoxic compounds. Resting microglial cells become activated, become profound antigen-presenting cells and start to secrete pro-inflammatory cytokines (IL-1β, TNF-α and IL-6) and mediators (reactive oxygen species, ROS), resulting in the recruitment of T-lymphocytes. Activated T-cells are capable of extravasating into the CNS, where they perform immune surveillance. Chronic elevation of myeloid suppressor cells (MSC), while not the primary cause of a disease, might contribute to the lack of recovery and further exacerbation of the disease conditions [40]. Cytokines like TGF-β1 are major regulators of the immune response, acting by exerting both anti-inflammatory and pro-inflammatory effects in a context-dependent manner. In neurological diseases Treg-derived systemic TGF-β1 inhibits T-cell-mediated disease, whereas locally increased TGF-β1 at the site of antigen presentation exacerbates disease. This is consistent with the chemotactic effects of TGF-β1 on T-lymphocytes and also its pro-inflammatory Th1 polarizing effects. In addition, TGF-β1 induces tissue repair and recruits microglia to the site of damage [41]. The combination of chronic and persistent neurological damage, increased antigen presentation by microglial cells, and suppressed immune regulation by increased numbers of Treg and myeloid suppressor cells, will result in activation and recruitment of pro-inflammatory Th1 cells and Th2 cells, promoting the development of autoantibodies directed against neural tissue. These factors strongly contribute to the development and aggravation of neurological disease. (In future histological analyses of such patients, T-cell subsets and cytokines, and B-cells and autoantibodies, should be detected and quantified.)

8.3 Involvement of Ca^{2+}/calmodulin-dependent kinase II (CaMKII)

The NFI autopsy reported that "*pentobarbital caused imbalance between oxygen supply and removal to the brain and the heart, resulting in a lack of oxygen, tissue damage and damage to the cardiac muscle, herniation of the brain, organ dysfunctions and death*". The results of this single case-study do not contradict the conclusion of the autopsy reports that death resulted from damage to the heart and the brain. In fact, a plausible explanation can be offered as to how the heart and brain were injured that agrees with the conclusions of the NFI report. It is postulated that exposure to organophosphates in the aircraft caused activation of Ca^{2+}/calmodulin-dependent kinase II (CaMKII), which resulted in heart damage and contributed to the subject's death. CaMKII is a multifunctional heteromeric serine/threonine protein kinase [42]. An early event in organophosphate-induced neurotoxicity is increased Ca^{2+} concentration in neuronal mitochondria [43], followed by enhanced autophosphorylation [44, 45], enhanced activity and increased mRNA expression [46–49] of CaMKII. Recent studies have shown that CaMKII (more specifically, CaMKIIδ) is a regulator of oligodendrocyte myelination and maturation [89]. Overexpression of CaMKIIδ engendered a decrease in the process network of oligodendrocytes. Thus, organophosphate-induced expression and activity of CaMKII results in release of myelin basic protein. CaMKII is also involved in the apoptotic death in early

stages of cardiac [49] and nervous system diseases [48]. This enhancement is also accompanied by an increase in apoptosis (Bax/Bcl-2 ratio and TUNEL-positive cells) associated with enhancement of CaMKII activity (CaMKII is a pre-apoptotic protein [50]). Activated CaMKII promotes heart failure by mediating pathological efforts of ischaemia reperfusion (IR) through induction of both apoptosis and necrosis. Activated CaMKII-induced cell death involves mitochondrial pro-death pathways [50]. This explanation is supported by the finding that inhibition of CaMKII attenuates cell death in the heart resulting from catecholamines, myocardial infarction or IR. Mitochondrial-triggered cell death results from CaMKII activated by Ca^{2+} overload or excess ROS production in the mitochondria [50]; the explanation is consistent with the autopsy report implicating imbalance of oxygen supply causing damage to the heart and the brain.

8.4 Brain weight

The autopsy report indicated that there were signs of fluid accumulation in the brain (his brain weighed 22% more than an average healthy adult brain). Increased water in the brain was a hallmark of brains, autopsied in the 1930s, of victims of TOCP poisoning (the "Ginger Jake"episode). Old autopsy reports contain phrases like "brain described as water-logged" [51] and "there was considerable oedema of the cortex and the meninges appeared thickened" [52].

9. DIFFERENTIAL DIAGNOSIS

For several years a debate has been ongoing regarding the cause of symptoms such as those exemplified by the present subject's health complaints and whether they are due to exposure to engine oil fumes or other factors [34]. Establishing a causal link with exposure is not easy; the main reason is there was (and still is) no onboard monitoring of aircraft cabin air contamination. This is in spite of the deep concerns that have been expressed over decades [1–5], and also in spite of the fact that many *ad hoc* detection tests, and well-resourced studies, have reported contamination [6–8]. Clinicians have to rely on their patients' histories to determine whether their symptoms relate to exposure. On the other hand, processes such as recall bias and attribution error can make patient testimony unreliable. To complicate matters further, patients usually see physicians long after exposure has ceased, when toxic substances may have been eliminated from the body, and results from routine medical investigation often fail to identify any abnormalities. Generally, clinicians are unaware of the possible toxic air contamination within aircraft. In order to find out the cause of the subject's chronic ill health and his eventual death, we carried out differential diagnosis or "detective toxicology" by considering his use of pentobarbital, alleged exposures to chemicals, the compendium of symptoms and complaints of the patient, routine medical evaluations, specialized tests, autopsy findings, autoantibody results and other possible nervous system diseases.

9.1 Involvement of pentobarbital in the subject's toxic burden, integrity of the blood–brain barrier (BBB) and neuronal cell death

9.1.1 Pentobarbital-induced toxicity

Pentobarbital has been implicated in the poisoning and sudden death of the subject because it was found in his body during autopsy. Pentobarbital can induce death when used in high doses (i.e., 10 g). Death occurs in 0.5 to 12% of cases; many are the result of deliberate attempts at suicide [53]. It is believed that poisoning often results from "drug automatism", which refers to the situation when a patient who could not go to sleep after the first or second dose becomes confused and, without being aware, ingests an overdose; if there is recovery there is no memory of having taken an additional dose. A study of 488 cases of intoxication classified approximately one fourth of these cases to be due to automatism [54]. The automatism cases showed a higher proportion of cerebral lesions than did the patients with suicidal intent, and the former were thus probably more disposed to a confessional state during mild intoxication. In the present case, histopathological assessment of the brain indicated the presence of cerebral lesions that are consistent with automatism and suggest that the subject's death might have been an overdose instead of suicide; this is in agreement with the absence of a suicide note [55]. Even if the subject did consume an overdose of pentobarbital that contributed to his death, it might be inferred that prior exposure to organophosphates caused severe cortical damage leading to drug automatism and the overdose [56, 57].

9.1.2 Effect of pentobarbital on the integrity of the blood–brain barrier

In the present investigation it is hypothesized that exposure to neurotoxic chemicals in the aircraft caused a breakdown of the blood–brain barrier (BBB) and neuronal death in the brain and spinal cord. The question whether these effects on the BBB and neuronal cells could have been caused by pentobarbital has arisen [56]. The post-mortem reports indicated that the subject had pentobarbital in his blood; however, it is not known for how long or how much; pentobarbital was found in the lower half only of a 2 cm hair, suggesting that he had not been using it for longer than about three months. It was present at a high enough concentration in the blood for the

post-mortem reports to conclude that it contributed to his death. We are unaware of published reports of the effect of either long-term low-level or acute large doses of pentobarbital on neuronal cells or the integrity of the BBB. In contrast, a sedative dose [57] of pentobarbital was found to protect both the BBB as well as neuronal cells from death, suggesting that when the BBB is disrupted, pentobarbital may be effective in protecting the BBB—an infusion of 20 or 50 mg/kg pentobarbital was reported to attenuate the degree of leakage of the BBB. Thus, when the BBB is disrupted, pentobarbital can play a significant rôle in decreasing the leakage.

9.1.3 Effect of pentobarbital on neuronal cell death

Barbiturates are known to prevent postischaemic cell death in selected vulnerable regions in the brain including CA1 pyramidal cells in experimental animals [58]. Moreover, inducing coma treatment with barbiturates has been an effective therapeutic method for cerebral ischaemia—pentobarbital resulted in complete protection against CA1 cell death in the hippocampus CA1 subfield on day 14 in accordance with previous reports [58]. Pentobarbital (50 mg/kg administered intraperitoneally) protected CA1 pyramidal cells from death. The neuroprotective mechanism of pentobarbital is generally considered to function via CNS depression, or through enhancement of $GABA_A$ receptor binding [59]. In the present case, it is concluded that neither the integrity of the BBB nor neuronal cell death had been affected or caused by pentobarbital.

9.2 Other diseases

Following the autopsy, the subject's pathology results were transiently considered as reminiscent of Guillain–Barré syndrome, which is an acute polyneuropathy affecting the peripheral nervous system [60]. Ascending paralysis (weakness beginning in the feet and hands and migrating towards the trunk) is the most typical symptom. It is usually triggered by an infection. With prompt treatment by intravenous immunoglobulins or plasmapheresis, together with supportive care, most sufferers will recover completely. Guillain–Barré syndrome is rare (one to two cases per 100 000 people annually). Unlike disorders such as multiple sclerosis (MS) [61] and amyotrophic lateral sclerosis (ALS, Lou Gehrig's disease) [62], it is a purely peripheral nerve disorder and does not in general cause nerve damage to the brain or spinal cord. It was discounted in the present case.

9.3 Organophosphate-induced nervous system injury

Aircrew and some passengers have been reporting ill health following air emissions for many years, the immediate effects being eye, ear, nose and throat irritation, respiratory problems, headaches, nausea and cognitive impairment, which usually recede on cessation of exposure, although a number of individuals report persistent chronic ill health including chronic fatigue, cognitive impairment, headaches, and muscle weakness [13, 19, 34]. Such individuals reported exposure to an environment containing organophosphates, insecticides (e.g., permethrin), carbon dioxide and other chemicals. It is most likely that their condition resulted from combined exposure to this mixture of chemicals instead of a single compound.

Numerous neurological diseases are characterized by neurodegeneration of unknown aetiology. On the other hand, nervous system damage may be caused by acute traumatic brain injury or following a single large chemical exposure that causes neurological deficits. It can also result from repeated or continuous low-level (chronic) chemical exposure, causing small increments of neural injuries that accumulate and ultimately result in neurological deficits. At some point a further single low-level exposure that in itself may be insufficient to result in the development of symptoms, but which occurs on the top of the pre-existing chronic exposure, may push neural injury above a threshold, leading to the development of symptoms of more or less severe neurological deficits. Symptoms and complaints reported in the present case are consistent with organophosphate-induced neurotoxicity (and OPIDN in particular) and affected by the following factors:

9.3.1 Chemicals

Although the identity or quantity of chemicals to which the subject was exposed is not known, recent studies have confirmed the presence of organophosphates in air samples aboard airplanes: TCP and tri-*n*-butyl phosphate (TBP) in aviation mechanics areas [63] and TCP and TOCP in cabin air on commercial and military aircraft [4, 64, 65]. Furthermore, a recent study was carried out to collect five-minute airborne sampling data during 100 flights on four aircraft types, B757, BAel47, A320 and A319, from September 2008 to February 2010 [66]. There were no confirmed fume events during any of the flights and the only reported symptoms among the 552 crew members were a headache/slight headache (four individuals). The detection limit for TOCP was 0.04 $\mu g/m^3$, for other TCP isomers 0.012 $\mu g/m^3$, and for TBP 0.8 $\mu g/m^3$. The highest five-minute average concentration of TOCP was 0.02 mg/m^3 on one flight. Detectable airborne concentrations of these chemicals were found for TOCP on 14 of the 100 flights, TCPs (non-TOCP, multiple isomers) on 23 and TBP on 73. It is not known how much higher air emission (chemical/oil fume) concentrations would have been during flights with documented/reported air emissions. In addition, events will vary in duration;

some are associated with a spill of oil into the system post-maintenance, for example, while others are longer lasting (ongoing leak). Recently, Lockridge and coworkers detected TOCP in six out of 12 jet airplane travellers by determining the stable adduct of the TOCP metabolite, cresyl saligenin phosphate, with plasma butyrylcholinesterase (BChE) [67]. They reported that none of the subjects exhibited or complained of toxicity symptoms. These reports suggest that low-level exposure to organophosphates during aircraft flights may be relatively common and contribute to flight crews' health complaints.

9.3.2 Route of exposure

Dermal exposure to organophosphates is more effective than oral administration in producing OPIDN: it took 64 daily oral doses of 1.0 mg/kg (total dose is 64 mg/kg) of the organophosphorus insecticide leptophos to cause OPIDN [68] but only 25 dermal doses of 0.5 mg/kg (total dose is 12.25 mg/kg) achieved the same effect in hens [69]; in other words, the total oral dose that caused OPIDN was 5 times that of the dermal dose. Although there are no data regarding inhalation exposure, this route is generally the most efficient for delivery of toxins to the nervous system. Also, daily dermal application of EPN [70] was 10 times more effective in causing OPIDN than oral administration [71].

9.3.3 Single and multiple exposures

Chronic or subchronic exposures to small doses of organophosphorus compounds are more effective in causing and more efficient in producing OPIDN than large single doses. Whereas the minimum single oral dose of TOCP that produced OPIDN in hens was determined to be 250 mg/kg, 36 daily oral doses of 0.5 mg/kg, totalling 18 mg/kg, induced OPIDN; in other words, the single dose that caused OPIDN was 14 times greater than that of divided doses producing the same effect [72]. Also, whereas the minimum oral dose of 200 mg/kg of leptophos [73] was required to produce OPIDN in hens, it took only 64 daily oral doses of 1.0 mg/kg to cause OPIDN [74], demonstrating that daily small oral doses of leptophos were three times as effective as a single oral dose for producing OPIDN [72]. Similarly, 36 small 0.5 mg/kg daily dermal doses of the organophosphorotrithopate, DEF (total dose is 18 mg/kg) caused OPIDN [74] in hens compared with a single 100 mg/kg dermal dose, indicating a sixfold increase [75]. Also, a daily dermal dose of 0.01 mg/kg EPN for 20 days (total dose 0.2 mg/kg) caused OPIDN [70], compared to a single 10 mg/kg dermal dose [76] indicating twentyfold increase.

9.3.4 Combined chemical exposure

Combined exposure to organophosphorus compounds increases their neurotoxicity because of competition for detoxifying enzymes, results in their persistence in the system, and more delivery to the brain. Inhalation or dermal application of the solvent methyl isobutyl ketone increased the severity of OPIDN induced by EPN, and decreased the threshold dose that causes OPIDN [77]. Also, propetamphos, an organophosphorus compound that is not capable of causing OPIDN, decreased the threshold oral dose of chlorpyrifos for inducing OPIDN [78]. Permethrin, a pyrethroid insecticide that has been used frequently in airplanes to control insects, has been found to increase OPIDN induced by malathion [79].

9.3.5 Symptoms and complaints

The subject's early symptoms were consistent with the cholinergic effects of organophosphates, particularly the relatively early reported episode of scintillating vision. The first sign of neurological deficits consistent with OPIDN occured in 2008 when he experienced a slow onset of numbness in hands and feet, creeping up as far as the elbows and knees. These symptoms are typical as the earliest manifestations of OPIDN [16]. In August 2011 he had paresthesia in both legs and both arms, which is a hallmark of OPIDN [16]. The symptoms reported to the medical doctors in the Netherlands are consistent with those of OPICN [9]. In Table 1, showing the summary of the subject's life-events, we have added remarks to elucidate such pointers. The time course of his complaints is consistent with organophosphate poisoning. The subject's description of symptoms relating to OPIDN, such as paresthesia and ataxia [14–18], are very specific and technical; he could hardly have invented them.

10. GENETIC VARIATION

It has been established that individual sensitivity to neurotoxicity induced by chemicals including organophosphates is genetically and environmentally controlled [90]. Furthermore, while a certain segment of the population tolerates exposure, other segments have diminished or nonexistent tolerance. This observation is related to the individual's genetic makeup. Following entry into the human body, an organophosphate undergoes metabolic processes including absorption, binding, distribution, storage, metabolic biotransformation and excretion [80].[2] In the liver the major enzymes that metabolize organophosphates into less

[2] By way of background, detoxification of organophosphate (OP) esters is carried out by specific enzymes mostly present in the liver and blood. In the present case, no attempt was made to find out if the subject had any genetic PON1 or BChE variants that might have contributed to his organophosphate-induced neurotoxicity.

toxic metabolites are the cytochrome P450 isozymes, via dearylation of aromatic OPs such as TCP and TBP, which are present in engine oil and hydraulic fluid, respectively. In blood, the first line of defence against organophosphate-induced toxicity are the enzymes paraoxonase1 (PON1) and plasma butyrylcholinesterase (BChE) [81, 82]. PON1 is a 354-amino acid plasma enzyme tightly associated with high-density lipoprotein particles (and also found in the liver) [70]; it is polymorphically distributed in human populations with the amino acid substitution glutamine → arginine (Gln → Arg) at position 192, which determines catalytic efficiency. Actual concentrations of PON1 vary by as much as fifteenfold among individuals with the same PON1(192) genotype [73].

BChE protects exposed individuals by acting as a scavenger, binding to organophosphates and subsequently hydrolysing them, resulting in their removal from circulation and leading to less of the OPs reaching neurotoxicity targets such as the brain [74]. Genetic variants known as atypical BChE have less ability to hydrolyse organophosphates; one in which the aspartate at position 70 is substituted by glycine is incapable of hydrolysing organophosphates [85, 86]. The usual homozygous enzyme (E^u, E^u) is 80% inhibited by dibucaine and is present in most of the Caucasian population. The heterozygous atypical enzyme (E^u, E^a) with 60% inhibition by dibucaine is present in about 4% of the population. The homozygous atypical enzyme (E^a, E^a) that is 20% inhibited by dibucaine occurs with an incidence of 0.04% to 0.6%. Individuals with such variants are likely to be more sensitive to organophosphate-induced neurotoxicity than individuals with the normal variant [87].

11. CONCLUSIONS

The complaints and symptoms of the subject in this case themselves represent very strong evidence for the source of illness. Although these symptoms may seem "non-specific" to a layman, or even a physician who is not familiar with organophosphate-induced neurotoxicity, their temporal occurrence and action are highly specific for neurotoxicity induced by organophosphates. Considering the fact that the subject was not a neurotoxicologist, it is safe to assume that he was not familiar with organophosphate-induced neurotoxicity [88], its literature, or even to his exposure to it, since he did not report any air emissions until questioned. Nevertheless, his symptoms were not only consistent with, but also identical to, known and documented effects of organophosphate poisoning. It can be surmised that his low-level chronic exposure to mixtures of organophosphates, which initially caused unnoticed small increments of nervous system injury, eventually surpassed the threshold level for symptoms to become evident. This is consistent with the subject not complaining initially; then, as time went by, he suffered symptoms of cholinergic neurotoxicity, followed by symptoms of OPIDN and finally OPICN.

Increased autoantibodies against nervous system-specific proteins are very strong evidence for nervous system injury. They indicate neuronal and glial degeneration consistent with OPIDN and OPICN. They also allow identification of the specific region(s) that is (are) injured, such as the spinal cord and cerebellum that are injured in OPIDN. Injury and degeneration of the cortex, hippocampus and cerebellum, as observed in the present subject [21], usually accompany OPICN [14].

The results of the present case study show that histopathological alterations in the brain confirm and validate the results of increased serum autoantibodies against brain-specific proteins. Both are consistent with organophosphate-induced neurotoxicity. Furthermore, the results offer an explanation for the inference that death resulted from damage to the heart and brain via organophosphate-induced activated CaMKII: exposure to the aircraft environment, for which there is evidence that it contains organophosphates, would have rendered the subject susceptible and predisposed to injury by pentobarbital. Organophosphates have been shown to cause overexpression, increased phosphorylation and increased activity of CaMKII [14, 17, 42], a pre-apoptotic protein that causes apoptotic cell death in both the brain and the heart.

In the absence of any competing diagnosis, the negative results of all other tests and examinations, and in the light of the discovery of very strong autoantibody markers for brain damage that is confirmed by the histopathological examination *post mortem*, one is drawn to the conclusion that the most likely cause of the subject's illness was organophosphate-induced neurotoxicity.

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