

**BEHAVIOURAL AND NEUROTOXIC EFFECTS OF
ALUMINUM HYDROXIDE AND SQUALENE ADJUVANTS
IN RELATION TO AMYOTROPHIC LATERAL
SCLEROSIS-GULF WAR ILLNESS**

by

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ABSTRACT

Gulf War Illness (GWI), more commonly known as Gulf War Syndrome, affects a significant percentage of veterans of the 1991 conflict, but its origins remain unknown. Associated with some cases of GWI are increased incidences of amyotrophic lateral sclerosis (ALS) and other neurological disorders. While many environmental factors have been linked to GWI, the role of the anthrax vaccine has come under increasing scrutiny. Among the vaccine's potentially toxic components are the adjuvants aluminum hydroxide and squalene. To examine whether these compounds might contribute to neuronal deficits associated with GWI, I developed an animal model for examining the potential neurological impact of aluminum hydroxide, squalene, or aluminum hydroxide combined with squalene. Young male colony CD-1 mice were injected with the adjuvants at doses equivalent to those given to U.S. military service personnel. All mice were subjected to a battery of behavioural (motor, cognitive and emotional) tests over a six-month period post injections. Following sacrifice, CNS tissues were examined using immunohistochemistry for evidence of inflammation and cell death. Behavioural testing showed motor deficits in the aluminum treatment group that expressed as a progressive decrease in strength measured by the wire mesh hang test (final deficit at 24 weeks: approx. 50%). Significant cognitive deficits in water maze learning were observed in the combined aluminum and squalene group (4.3 errors/trial) compared to controls (0.2 errors/trial) after 20 weeks. Apoptotic neurons were identified in aluminum injected animals and showed significantly increased activated caspase-3 labeling in lumbar spinal cord (255%) and primary motor cortex (192%) compared to controls. Aluminum treated groups also showed significant motor neuron loss (35%) and increased numbers of

activated astrocytes (350%) in the lumbar spinal cord. Preliminary results from Iba-1 staining showed microglial proliferation in lumbar spinal cord of aluminum treated animals. Morin staining detected the presence of the aluminum within the cell body and/or nucleus of neurons in this same area. The findings suggest a possible role for the aluminum adjuvant in some neurological features associated with GWI and possibly an additional role for the combination of adjuvants.

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LIST OF ABBREVIATIONS

ALS = Amyotrophic lateral sclerosis

ALS-PDC = Amyotrophic lateral sclerosis – parkinsonism dementia complex

AVA = Anthrax vaccine absorbed

AVIP = Anthrax vaccine immunization program

CHAT = Choline acetyltransferase

CNS = Central nervous system

DAPI = 4',6 diamidino-2-phenylindole

DOD = Department of Defence

DOVA = Department of Veteran Affairs

DPT = Diphtheria, pertussis, and tetanus.

EDTA = ethylene diamine tetraacetic acid

FDA = Food and Drug Administration

FITC = Fluorescein-5-isothiocyanate

FJB = Fluoro-Jade B

GFAP = Glial fibrillary acidic protein

GWI = Gulf War illness

IOM = Institute of Medicine

NEUN = Neuronal nuclei

NFH = Neurofilament, heavy

ROI = Region of interest

SA = Squalene antibodies

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INTRODUCTION

History and Description of Gulf War Illness

Gulf War Illness (GWI), popularly termed “Gulf War Syndrome”, is a spectrum of disorders amongst veterans of the Gulf War (1990-91) characterized by a group of variable and nonspecific symptoms such as fatigue, muscle and joint pains, emotional disorders, posttraumatic stress reactions, headaches, and memory loss (Haley et al., 1997; Fukuda et al., 1998). Between 1990 and 1991, the U.S. armed forces sent 697,000 soldiers to the Persian Gulf (Institute of Medicine, 1995); in the years following the Gulf War conflict, a significant population (70%) of soldiers who were active during operation Desert Storm began reporting generalized ill health with accompanying multiple physical and mental symptoms (Haley et al., 1997).

Since the end of the Gulf War in 1991, several studies conducted on Gulf War veterans by the U.S. DOD, the U.S. Department of Veteran Affairs (DOVA) and the U.K. Gulf War Research Illness Unit have established and confirmed a strong link between Gulf War-era service and the occurrence of GWI (Haley et al., 1997; Hom et al., 1997; Unwin et al., 1999; Kang et al., 2002; Wolfe et al., 2002; Dyer, 2004). All of these studies concluded that military personnel who participated in the Gulf War have a higher self-reported prevalence of medical and psychiatric conditions than those military personnel who were not deployed.

The first epidemiologic, clinical, and laboratory research that found a clear effect of the "Gulf War syndrome" and related neurological illnesses in Gulf War veterans was conducted by Dr. Robert Haley and colleagues at the University of Texas Southwestern Medical Center in March 1994 (in part funded by the Perot Foundation and U.S. Department of Defense (DOD) (Haley et al., 1997). The objectives of the research were to define new or unique clinical syndromes among Gulf War veterans, determine their causes, identify areas of damage or dysfunction in the brain and nervous system responsible for the symptoms, develop a cost-effective battery of clinical tests that could diagnose the illness, search for underlying genetic traits that might predispose to the illness, and perform clinical trials of promising treatments. Haley recognized that no controlled study had been done to compare ill and healthy veterans to define the illness and test risk factors. To search for syndromes among Gulf War veterans, Haley and his group began epidemiological studies on 249 veterans of the 24th Reserve Naval Mobile Construction Battalion (RNMCB-24) from five southeastern states. Of 249 participants, 175 (70%) reported having had serious health problems that most attributed to the war; 74 (30%) reported no serious health problems. Initial analyses of the epidemiologic survey identified primary six syndromes and demonstrated that primary syndromes 1-3 were associated with exposure to various combinations of cholinesterase-inhibiting chemicals (Haley et al., 1997). A 'syndrome' is a group of signs and symptoms that occur together and characterize a particular abnormality (Merriam-Webster Medical Dictionary, 2006). Table 1 below shows dichotomized (derived using factor analysis to disentangle different

meanings of ambiguous symptoms) syndrome indicators that identified six syndromes (Haley et al., 1997).

Table 1

The six syndrome factors of Gulf War illness		
Dichotomized syndrome		Symptoms
1.	“impaired cognition”	distractibility, difficulty remembering, depression, middle and terminal insomnia, daytime sleepiness, slurred speech, confusion, migraine-like headaches.
2.	“confusion-ataxia”	problems thinking and reasoning, confusion and disorientation, dizziness, imbalance and vertigo, sexual impotence.
3.	“arthro-myo-neuropathy”	joint and muscle pains, muscle weakness, muscle fatigue, and tingling or numbness of extremities.
4.	“phobia-apraxia”	nausea, faintness, chest discomfort, anxiety, difficulty in controlling hands or arms; and tingling or numbness of the trunk and groin.
5.	“fever-adenopathy”	fever with or without night sweats and swollen glands in diverse locations (authors did not specify).
6.	“weakness-incontinence”	Difficulty controlling bowels and bladder; standing from a chair; tingling or numbness in the face, tongue, and lips; and dyspareunia (difficult or painful sexual intercourse)

Haley and colleagues concluded that their findings supported the hypothesis that clusters of symptoms of many Gulf War veterans represent factor analysis-derived¹ syndromes that appear to reflect a spectrum of neurologic injury involving the central, peripheral, and autonomic nervous systems.

¹ The analytical process of transforming statistical data, as measured by ‘scores’ assigned to various health symptoms reported, into linear combinations of usually independent variables.

To further explore the nature of these syndromes, Hom et al. conducted a study to investigate the neurocognitive and psychological functions of veterans who reported Gulf War-related symptoms. After intensive and sophisticated neurological testing of several Gulf War veterans, evaluation of the findings in one group of 43 patients, including 23 with the 'syndrome' and 20 without (healthy controls), Hom and his group were unable to reach a diagnosis of a known syndrome in any of them (Hom et al., 1997). The 23 veterans with factor-derived syndromes have significantly more neuropsychological evidence of brain dysfunction than those lacking any of the syndromes. Analyses of the psychological tests showed some veterans with GWI were not suffering from combat stress, post-traumatic stress disorder, depression, malingering, or other psychological disorders. They concluded that some of the ill veterans have experienced neurological injury due to unknown toxins resulting in chronic neuropsychological impairment related to their service in the Gulf War, and the 3 factor-derived symptoms identified among Gulf War veterans appear to represent variants of a generalized injury to the nervous system (Hom et al., 1997). The results from these epidemiological, psychological, and neurological studies first began to identify 'Gulf War syndrome' as a unique illness that was exclusive to Gulf War veterans.

In addition to the correlation studies performed by Dr. Haley's group in 1997, the Iowa Persian Gulf Study Group (IPGSG) conducted an independent and larger study to assess the prevalence of self-reported symptoms and illnesses among military personnel deployed during the Gulf War. The study involved 3,695 military personnel from the

state of Iowa and involved both deployed and non-deployed servicemen. Gulf War military personnel reported significantly higher prevalence of symptoms of depression, posttraumatic stress disorder (PTSD), chronic fatigue, cognitive dysfunction, bronchitis, asthma, fibromyalgia, alcohol abuse, anxiety, and discomfort during sexual intercourse compared to non-Gulf War military controls (The Iowa Persian Gulf Study Group, 1997). Assessment of health-related quality of life also revealed diminished mental and physical functioning scores for Gulf War military personnel (The Iowa Persian Gulf Study Group, 1997).

In recent years, numerous studies have begun to explore the extent of neurological injury in patients with GWI using multiple imaging techniques. In 2000, Haley and colleagues used magnetic resonance spectroscopy (MRS) to test for neuronal brain damage in 22 Gulf War veterans with one of the foremost three factor analysis-derived syndromes. They discovered that the N-acetylaspartate-to-creatine (NAA/Cr) ratio, which reflects functional neuronal mass (Ross et al., 1992), was significantly reduced in the basal ganglia and brainstems of Gulf War veterans than those same structures in control subjects (Haley et al., 2000). NAA has previously been conceptualized as a marker of intact neurons in numerous neurological and psychiatric disorders (Barker, 2001). This conceptualization is derived in that more NAA could conceivably be linked to more neuronal mass (e.g., dendritic arbor, increased neuronal fraction), which in turn should underlie intelligent behavior, although the exact mechanism by which NAA is related to neuronal functioning, and hence broad measures of cognition, is unknown.

In a follow-up study using 27 Gulf War veterans, Haley and colleagues found that the reduction in functioning neuronal mass in the left basal ganglia of veterans with GWI appeared to have central dopamine production in a lateralized pattern, which is comparable with laterality of the effects of neuronal damage upon control of central dopamine activity demonstrated previously in rodent experiments (Carlson et al., 1996; Louilot and Choulli, 1997). Thus, they concluded that the neurological illness might be, in part, related to injury to dopaminergic neurons in the basal ganglia (Haley et al., 2000). A separate study in 2004 by Menon et al. elaborated on these findings by examining the hippocampus of patients with Gulf War syndrome. The subjects included 21 veterans, 10 of whom had GWI, 15 of whom served in the Gulf War while the remaining six had served in Vietnam. Using similar MRS techniques, they found that the NAA/Cr ratio, from both the left and right hippocampus of the GWI group was significantly lower than that of the entire control group or the unaffected GW control group (Menon et al., 2004). A more recent study (Vythilingam et al., 2005) used magnetic resonance imaging (MRI) to examine volumes of several structures including the hippocampus, temporal lobe, and whole brain in addition to evaluating short-term verbal memory. The results showed the head of the hippocampus was the only subregion that was significantly smaller in Gulf War veterans with post-traumatic stress disorder (PTSD) than in healthy civilians, but all Gulf War veterans had significantly smaller whole hippocampal volumes and lower scores on verbal and visual retrieval compared with healthy civilians (Vythilingam et al., 2005).

Controversy over the existence and classification of Gulf War-related illnesses remains today; however, the correlation studies (as previously described) on Gulf War veterans and ill health provide evidence for the existence of GWI. Epidemiological studies (mentioned earlier) on symptomatic Gulf War veterans have begun to classify GWI as a disorder with neurological origin. The evidence for a new illness present in Gulf War veterans is mounting and GWI has gained greater acceptance as a genuine disease with neurological components, one of which is ALS.

Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease, is a chronic, progressive neurodegenerative disorder characterized by motor neuron degeneration of spinal cord (lower and upper motor neurons), motor cortex, and brainstem as well as variable involvement of the descending motor tracts and other neurons such as astrocytes and microglia (Eisen and Krieger, 1998). Classical ALS symptoms include muscle weakness in the hands, arms, legs, weakness of speech muscles, twitching (fasciculations) and cramping of muscles in hands and feet, impairment of arms and legs, difficulty in projecting one's voice, and in more advanced stages, shortness of breath, difficulty in breathing and swallowing (i.e. dysphagia) (Rowland, 1998; Zoccolella et al., 2006a). In the end stages of the disease, diaphragm palsy occurs resulting in respiratory failure and death ensues on average within 3 to 5 years of symptom onset (Czaplinski et al., 2003; Strong, 2004).

ALS Pathology

The principle characteristics of neuropathology of ALS are loss of motor neurons in the anterior horns of the spinal cord and in the motor nuclei of the brain stem that results in secondary atrophy of the corresponding muscles (amyotrophy) (Ikemoto et al., 2000). Patients develop variable hyperreflexia, clonus, spasticity, and limb or tongue fasciculations (Schieppati et al., 1985; Ince et al., 1998). Amyotrophy is followed by paralysis that is attributed to the death of the lower motor neurons. Typically, neuronal and axonal loss is followed by demyelination and shrinkage of cells in the corticospinal tract, but ALS also affects motor neurons that innervate the muscles (Eisen and Krieger, 1998). The sclerosis, or hardening, of ALS involves only the lateral columns and anterior horns of the spinal cord, or corticospinal tracts that result in progressive muscle atrophy that starts in the limbs. Wallerian degeneration, a sequence of axonal and myelin degeneration (of the axon distal to the site of injury), of corticospinal and corticobulbar tracts has been documented in postmortem examination and demonstrated by MRI (high-intensity T2 lesions in frontal lobes) (di Trapani et al., 1986; Udaka et al., 1992). Interestingly, the involvement of Onuf's nucleus, an area in the sacral spinal cord involved in the maintenance of urinary and faecal continence, in ALS pathogenesis is not fully understood, as in some cases it is spared. Some cases of ALS have the appearance of conglomerate inclusions or Bunina bodies (which are cystatin C-containing inclusions) and neuron atrophy in Onuf's nucleus; whereas in other cases, motor neurons in this structure appear to survive and are spared, although this structure is less vulnerable than other motor ganglia (Kihira et al., 1991).

Motor neuron death is typically accompanied by astrocytic and microglia proliferation that surrounds both upper and lower motor neurons, with some reactive gliosis occurring in the lateral descending corticospinal tracts including those leading into grey matter (Nagy et al., 1994; Schiffer et al., 1996; Hirano, 1996). Recent evidence has begun to identify active roles for astrocytes and other glial cells in the stimulation and propagation of motor neuron loss in ALS. Reactive astrocytes have been observed to cause mitochondrial damage and decrease glutamate transport (Ridet et al., 1997), instigate abnormal regulation of glutamate-induced excitotoxicity (Eisen and Krieger, 1998), and trigger apoptosis in motor neurons (Barbeito et al., 2004). Activated microglia can secrete pro-inflammatory peptides and nitric oxide (NO) that induce astrocytosis or aggravate neuronal damage, which serves to perpetuate or amplify the pathological processes in ALS (Giulian and Baker, 1986; Hall et al., 1998). There is also evidence for neurofilament (NF) accumulation in motor neurons of the spinal cord from ALS patients (Mendonca et al., 2005). The build-up of neurofilaments, classified as light (NF-L), medium (NF-M), or heavy (NF-H) subunits, can lead to aggregate formation in the neuron and alter NF transport resulting in axonal degeneration and the loss of α -motor neurons (Tsang et al., 2000; Mendonca et al., 2005).

Forms of ALS

Several distinct forms of ALS have been recognized: a common, sporadic form (sALS); a familial form (fALS) typically having autosomal dominant transmission, as well as a variant seen in three loci in the Western Pacific sometimes associated with forms of

parkinsonism and dementia (ALS-parkinsonism-dementia complex (ALS-PDC) (Hirano et al., 1966; Kurland, 1988; Hirano, 1992). Approximately 95% of all ALS cases are sporadic in that there is no clear genetic link or traceable family history (excluding environmental exposures) (Kato et al., 1999). The most thoroughly studied cluster of ALS-PDC is on Guam. Clinically, the ALS component of Guamanian ALS-PDC is practically indistinguishable from sALS and presents with fasciculations as well as lower and upper motor neuron signs (Murakami, 1999). The primary difference between ALS-PDC from that of sALS is the occasional PDC component and widespread appearance of neurofibrillary tangles (NFT) in various CNS regions in the former.

The number of individuals diagnosed with ALS is between 1.2 and 1.8/100,000 in worldwide and it affects males more than women but is race independent (Strong and Rosenfeld, 2003). The ratio of males to females in fALS is 1:1 but 1.5-2:1 in sALS, although there appears to be male predominance in younger onset cases for both forms (Haverkamp et al., 1995; Worms, 2001). The mean age of onset for sALS is 59 years and the age of onset of fALS is normally distributed about a mean of 45.7 years, although younger cases (rare before age 20) of ALS have been reported in each form (Strong et al., 1991; Worms, 2001).

Both genetic and environmental factors have been proposed in the pathogenesis of ALS. In 5-10% of all ALS patients, fALS is identified, and approximately 20% of these fALS cases have mutations in the gene coding for the antioxidant enzyme, superoxide

dismutase (SOD) (Armani et al., 1987; Siddique et al., 1996). sALS is thought to arise from the action of unknown environmental toxins, potentially acting in synergy with various susceptible genes (Shaw and Wilson, 2003; Wilson et al., 2005; Kriscenski-Perry et al., 2002). With the Gumanian variant, ALS-PDC is associated with the consumption of exogenous neurotoxin found in the seeds of a local variety of cycad (*Cycas micronesica* K.D. Hill) (Kurland, 1988).

Amyotrophic Lateral Sclerosis – Gulf War Illness

Recent studies have also established a correlation between Gulf War service and a neurological cluster of Amyotrophic Lateral Sclerosis – Gulf War Illness (ALS-GWI) (Charatan, 2002; Horner et al., 2003; Haley, 2003; Weisskopf et al., 2005). In a study by Haley (2003), classical ALS symptoms such as muscular weakness, muscle wasting, impaired speech and swallowing, difficulty in breathing, and fasciculations (in some patients) developed in Gulf War veterans years after they first developed symptoms of “Gulf War illness” (“undiagnosed illness”) during or soon after they returned from the Gulf War (Haley, 2003). The most common “Gulf War illness” symptoms included memory/concentration problems, chronic fatigue, sleep disturbances, chronic pain, vertigo attacks, chronic diarrhea, chronic fever, night sweats, personality changes, and skin rashes. Seventeen of the 20 servicemen diagnosed with Gulf War illness and definite ALS were less than 45 years of age with the youngest 20 years old. All 20 of these patients presented signs of upper and lower motor neuron degeneration in the bulbar

region and at least two other spinal regions. None of these patients had a family history of ALS or other neurodegenerative disorders. Due to the overlapping symptomatology seen in ALS-GWI and classical ALS, GWI can be partially described as a neurological illness that may carry an ALS component.

Horner and colleagues conducted a larger scale study to determine if U.S. Gulf War veterans indeed have an elevated rate of ALS (Horner et al., 2003). A nationwide case study was performed to identify all occurrences of ALS for the decade period since August 1990 among active duty members of the military (as opposed to reserve personnel). 107 confirmed cases of ALS were identified among approximately 2.5 million eligible military personnel. When standardized to the average 1990 U.S. general population, the average annual rate of ALS among non-deployed military population was 1.4 per 100,000 persons per year as compared to the generally accepted overall population rate of 1 to 2 cases of ALS per 100,000 in the U.S. (Horner et al., 2003). However, the occurrence rate of ALS among the deployed military population was 3.6 per 100,000 persons per year when also standardized to the 1990 U.S. general population. In addition, the incidence rate of ALS between military groups was also examined and their findings identified the greatest elevated risk of ALS among deployed personnel in the Air Force and Army divisions, experiencing a significantly elevated relative risk of ALS of approximately two or greater. Elevated, but non-significant, risks were observed for deployed Reserves and National Guard, deployed Navy, and deployed Marine Corps.

A recent study by Weisskopf and colleagues in 2005, helped confirm the increased mortality rates of ALS among the military population. A cohort of over 500,000 men from 50 states, Washington, D.C. and Puerto Rico was investigated and participant follow up was conducted from 1989 through 1998 for ALS mortality. The study identified 280 deaths from ALS among 126,414 men who did not serve in the military and 281,874 who did serve, and revealed that men who served in the military had an increased death rate from ALS compared to those who did not serve (Weisskopf et al., 2005). The increased risk of ALS, in this study, appeared to be largely independent of the branch of service and the time period served.

According to these nationwide studies, deployed veterans of the Gulf War are more than *twice* as likely to develop ALS as non-deployed veterans and the civilian population (Samson, 2002). Unlike ALS-GWI, overall GWI, however, does not appear to distinguish between troops who were deployed to the Gulf versus those who were not (Steele, 2000). The most unique feature of this new ALS cluster is that the victims are younger than typical ALS patients (Haley, 2003).

Both ALS clusters offer the possibility to identify causal environmental and/or genetic factors involved in sALS. In regard to ALS-GWI and GWI in general, epidemiological studies have suggested several potential environmental factors such as exposure to depleted uranium (Fulco et al., 2000; Shawky, 2002), nerve gas (e.g., sarin, soman, tabun, VX) (Sartin, 2000; Kalra et al., 2002), organophosphates (Abou-Donia et al., 1996; Kurt,

1998), N,N-diethyl-*m*-toluamide (DEET) (Haley and Kurt, 1997), pyridostigmine bromide (PB) (Shen, 1998; Moss, 2001), vaccines (Hotopf et al., 2000), heavy metals (Ferguson and Cassaday, 2001-2002), gene susceptibility (paraoxonase; PON1) (Haley et al., 1999), and bacterial infections (e.g. *Helicobacter pylori*, mycoplasma) (Taylor et al., 1997; Nicolson et al., 2002).

The Anthrax Vaccine

In recent years, increased scrutiny has focused on vaccines in relation to GWI, in particular the anthrax vaccine absorbed (AVA) (Nass, 1999), largely due to the observation that non-deployed but vaccinated U.S. troops have developed GWI symptoms identical to those who were deployed (Steele, 2000). Soldiers from the United Kingdom who also received AVA showed increased psychological distress and chronic fatigue compared to control cohorts, (Unwin et al., 1999). In contrast, Hunter et al. (2004) released a study that examined health effects of Canadian soldiers post anthrax vaccination, but found no apparent link to the AVA vaccine and adverse health effects. Notably, however, the latter study only monitored health outcomes for a maximum of 8 months post vaccination; typically patients with Gulf War illness did not express GWI symptoms until years after the war. French soldiers participating in the war did not receive the AVA vaccine but do show some GWI related disorders (respiratory, neurocognitive, psychological, and musculoskeletal), but no ALS symptoms were reported (Salamon et al., 2006).

The anthrax vaccine was first developed at Microbiological Research Establishment (MRE), HPA-PD's predecessor, in the late 1950s to early 1960s and first became available for human use in 1963 (Turnbull, 1991). This vaccine was found to be safe and effective in preventing anthrax infection (cutaneous rather than inhalation anthrax) in mill workers in the late 1950s. The vaccine is manufactured using an avirulent strain of *Bacillus anthracis* originally isolated by Sterne in 1937. A UK product license (PL 1511/0037) was granted for the vaccine in 1979. A purer, more potent form of the anthrax vaccine was later developed which the Food and Drug Administration (FDA) licensed in November 1970 (Turnbull, 1991). It is still used today and is currently manufactured by Bioport Corporation (parent company Emergent BioSolutions). The U.S. product insert for the anthrax vaccine in 1990 lists the final product as a sterile product made of the recombinant protective antigen (rPA) from an avirulent non-encapsulated strain of *Bacillus anthracis* and formulated to contain 2.4mg aluminum hydroxide (equivalent to 0.83 mg aluminum) per 0.5 cc dose; formaldehyde, in a final concentration not to exceed 0.02%; and benzethonium chloride, 0.0025%, added as preservatives (product license no. 99, Bureau of Laboratories, Michigan Department of Public Health, Lansing, MI, USA).

The anthrax vaccine, in common with many other vaccines in wide usage, contains one chemical of particular interest from a neurological perspective: aluminum. A second chemical, the lipid polymer squalene (a precursor to cholesterol and phytosterol) has been

found in some lots of AVA (Plaisier, 2000), however, manufacturers of the AVA vaccine, along with DOD and other government agencies, deny that squalene was ever part of the formulation of AVA during the period in question. Antibodies to squalene have been demonstrated in many personnel expressing GWI (Asa et al., 2000). The origin of presumed squalene acting to trigger antibody formation remains uncertain.

The AVA vaccine has been critiqued on both safety and efficacy grounds (Nass, 2002; Schumm et al., 2002ba; Nass et al., 2005) and concerns have been raised that the Institute of Medicine (IOM) ignored evidence from studies that implicate vaccine involvement in the epidemiology of GWI (Schumm et al., 2002a). A recent publication has raised additional concerns about the long-term safety of the anthrax vaccine (Schumm et al., 2005).

Adjuvants: Aluminum Hydroxide and Squalene

An adjuvant is a substance that is added during production to non-specifically increase the body's immune response to an antigen (Brewer, 2006). Aluminum salts were first identified as adjuvants over 70 years ago and currently aluminum, in various forms (aluminum hydroxide, aluminum phosphate and aluminum sulfate), is the most common currently licensed adjuvant and is generally regarded by industry and the regulatory agencies as safe (Lindblad, 2004a). However, in spite of their long history of widespread use, the physicochemical interactions between aluminum compounds and antigens are

relatively poorly understood and their underlying mechanisms remain relatively unstudied (Lindblad, 2004b).

Previous studies have found no adverse or long-term health effects of these adjuvants (Baylor et al., 2002; Kanra et al., 2003; Jefferson et al., 2004) and the Food and Drug Administration (FDA) agency has continued its long-standing approval. However, aluminum in general has been shown to be neurotoxic under some conditions (Crapper et al., 1973; Kawahara et al., 2001) and adjuvants in particular have previously been implicated in neurological disease (Garruto et al., 1989; Wagner-Recio et al., 1991; Bilkei-Gorzo, 1993). Table 2 below shows findings from previous studies that treated animals with aluminum hydroxide and examined the potential impact on the CNS.

Table 2

Summary of aluminum hydroxide studies examining CNS					
Animal	Age	Dose	Injection Type	Result	Reference
Female NIH mice	4 week	315-335µg	i.p.	Significantly elevated levels of Al in brain	Redhead et al., 1991
Male and female Long Evan rats	2 month	100 or 300mg/kg/day	oral	Significantly reduced learning ability and elevated levels of Al in brain	Bilkei-Gorzo, 1993
Male Swiss albino mice	Not stated	~20µg/day	oral	Significantly elevated levels of Al in brain, kidney and liver.	Sahin et al., 1994
Pzh:SFIS mice	Not stated	1.0mg every 2 weeks or 0.1mg 5 days/week	i.p.	Significantly elevated levels of Al in liver and tibia (bone), but not in brain.	Fiejka et al., 1996

Squalene has been intensively investigated as a potential adjuvant with some reports failing to find any significant health outcomes (Benisek et al., 2004; Suli et al., 2004; Gabutti et al., 2005). The potential toxicity of squalene is controversial, however, and some reports have demonstrated both neuropathology (Gajkowska et al., 1999) and inflammatory responses (Carlson et al., 2000) in animal tests, albeit at very high concentrations. LD₅₀ values (for subcutaneous injection) for either aluminum hydroxide or squalene have not been published to date to the best of my knowledge (J.T. Baker Material Safety Data Sheets) (for chemical structure see appendix 1).

Rationale

Currently, there is no known cause or trigger for Gulf War "illness" or "syndrome" and it is difficult to identify contributory factors given the immense list of variables and exposures involved in the Gulf War. As such, the role(s) of physiological and psychological stress, genetic predisposition, and environmental exposures on and off the battlefield (nerve gases, pesticides, vaccinations, etc.) and how these all interplay further complicate the issue. However, the chronic onset of multi-systemic symptomology of GWI, the sporadic nature amongst a diverse military population, and the observed incidence rate of sALS in young Gulf War veterans strongly suggest an environmental role or trigger for GWI.

There currently exists a plethora of environmental agents that have become suspect in development of Gulf War illness including depleted uranium, radiation, nerve gas, pesticides, organophosphates, heavy metals, bacterial infections and vaccines (see introduction above). Given the controversies surrounding the anthrax vaccine absorbed (AVA) and its known vaccine adjuvants as a strong candidate for having a possible role in the development of ALS-GWI, I have decided to investigate whether the contents of the anthrax vaccine administered to soldiers during the Gulf War, specifically the adjuvants contained in the vaccine, contributed to the development of ALS-GWI.

Hypotheses and Objectives

The hypotheses to be tested are: 1) Aluminum hydroxide or squalene, alone or in combination, will induce behavioural and pathological CNS outcomes resembling those seen in ALS-GWI. 2) Any such deficits will be progressive.

To test these hypotheses, I designed an experiment to provide an accurate multi-level analysis of the potential impact of aluminum hydroxide and squalene on the central nervous system over extended time periods in an outbred strain (CD-1) of young male mice. In this experiment, juvenile CD-1 male mice were injected subcutaneously with either aluminum hydroxide, squalene, aluminum hydroxide plus squalene, or phosphate buffered saline (control), and tested on various motor and cognitive tasks. Following sacrifice, CNS samples were examined for any evidence of pathology. The conditions chosen in this model system were intended to mimic the administration of AVA to young, predominantly male, U.S. and other coalition military service personnel.

To examine if the adjuvants aluminum hydroxide or squalene contribute towards the development of ALS-GWI, it is important to examine if: 1) mice injected with these adjuvants (with minimal doses) develop cognitive deficits, motor problems and cellular pathology that resembles ALS-GWI, 2) whether alone or in combination these adjuvants have a more active role (if any) in the development of symptom onset and severity, and 3) whether the impact (if any) of these adjuvants is persistent over time.

METHODS

Animals

Young adult CD-1 male mice were used in the study (3 months old; weight approximately 35g at experiment onset). Younger animals were deliberately chosen to mimic the age of service of most military personnel during the Gulf War (Haley, 2003). An outbred strain of mice was chosen because they generally have high disease resistance and are genetically more variable, which more accurately mimics what one would find in humans and the military test population. Four treatment groups were used: control (n=10) injected with saline/phosphate buffered solution (PBS), aluminum hydroxide (n=11), squalene (n=10), and aluminum hydroxide plus squalene (n=10).

Housing and Diet

All animals were housed solitarily at the Jack Bell Research Centre animal care facility (Vancouver, B.C., Canada) in clear plastic cages each containing Bed O' cobs corn cob bedding (The Andersons, Maumee, OH), a 5 x 5 x 0.5 cm square of cotton bedding, a metal ring, and a plastic tube (or dome) for shelter. An ambient temperature of 22 +/- 1°C and a 12/12hr light cycle with lights on at 06:00h. All mice were fed Purina® mouse chow and water *ad libitum* throughout the experiment. To monitor weight changes due to adjuvant injections, all mice were weighed weekly.

Adjuvants

Alhydrogel[®], an aluminum hydroxide ($\text{Al}(\text{OH})_3$) gel suspension was used as a source of aluminum hydroxide. Alhydrogel[®] (which is used in the AVA vaccine) is manufactured by Superfos Biosector a/s (Denmark). MPL[®] + TDM + CWS (Monophosphoryl Lipid A, synthetic Trehalose Dicorynomycolate, and cell wall skeleton of mycobacteria), is a commercial squalene ($\text{C}_{30}\text{H}_{50}$) containing adjuvant was manufactured by Corixa Corporation (Seattle, USA). Both adjuvants were supplied by SIGMA, Canada.

Aluminum: To calculate approximate human dosages of aluminum hydroxide and squalene for our experiments, we used the following information. According to product data sheets from the Michigan Biologic Products Institute (MBPI, Lansing, Michigan, USA; the AVA manufacturer during the Gulf War), a single dose of AVA vaccine contains 2.4 mg of aluminum hydroxide (equivalent to 0.83mg of aluminum). Based on an average human body weight of 70-80 kg, the amount per kg body weight is approximately 30-34 $\mu\text{g}/\text{kg}$. Soldiers or civilians receiving a range of 1-4 doses of the vaccine would have received between 30-34 $\mu\text{g}/\text{kg}$ (1 injection) up to 120-136 $\mu\text{g}/\text{kg}$ if 4 injections were received.

Squalene: As noted above, both Biopart Corporation and the Michigan Biological Products Institute deny the addition of squalene in AVA formulations, past or present. Our calculations are therefore based on current vaccines in use outside the United States that employ the squalene containing adjuvant oil emulsion, MF59. This adjuvant in

experimental influenza vaccines (Chiron Corporation, Emeryville, CA, USA) uses a concentration of 5% squalene. Based on the total volume of the MF59 injection (0.5 ml), this would be equivalent to 0.025ml of squalene. Again, based on an average 70-80 kg human, the amount per injection would be approximately (0.31-0.35 μ g/kg) for one injection, as much as (1.24-1.40 μ g/kg) for a full series of 4 injections.

The adjuvant injections in mice were calibrated based on average animal weight for 3-month-old male CD-1 mice (approximately 35g). We chose to do two injections as an average based on U.S. DOD usage (1 to 4 doses) during the Gulf War in 1991. Based on the human values cited above, mice receiving aluminum hydroxide received two doses of 50 μ g/kg (suspension) in a total volume of 200 μ L sterile PBS (0.9%). The mice in this experiment would therefore have received 100 μ g/kg versus a probable 68 μ g/kg in humans. Mice receiving squalene got the equivalent dose of 2% squalene suspension (MPL[®] + TDM + CWS) in PBS for a total of (0.24-0.28 μ g/kg) over two injections compared to the likely human dose (0.62-0.71 μ g/kg) at 5% squalene over two injections. Mice in the aluminum hydroxide + squalene group had both adjuvants administered in the same PBS volume. Controls were injected with 200 μ L PBS.

Vaccination

The injection site for human administration is typically subcutaneous over the deltoid muscle. For injections in mice we used a subcutaneous injection into the loose skin behind the neck (the "scruff") to minimize discomfort and for ease of injection.

Animals received two injections (two weeks apart) of aluminum hydroxide, squalene, aluminum hydroxide and squalene, or PBS. This vaccination protocol mimicked the anthrax vaccine dose schedule set by the Anthrax Vaccine Immunization Program (AVIP) except for the location of the injection.

Behavioural Tests

Mice were subjected at regular intervals to specific behavioural tests, including wire mesh hang (3x/week), open field (1x/week), and water maze (1x/week), leg extension (3x/week), and gait length (2x/week) over a period of six months (24 weeks) post injections. The order in which the animals were tested was randomized for each trial. In all behavioural tests and histological assays the experimenters were blind to the identity of treatment groups of the animals or samples.

Motor Tests

Wire Mesh Hang

A wire mesh hang test was used 3x/week to test for muscular grip strength and endurance (Crawley, 2000; Sango et al., 1996; Wallace et al., 1980). The wire mesh hang consisted of a 6-inch wire mesh that was suspended 40 cm above a padded surface, high enough to discourage the mouse from falling and low enough to not cause injury if the mouse fell (Fig. 1A). Mice were placed onto the wire grid and inverted for a maximum period of 60 seconds; normal mice can hang inverted for several minutes. Latency to fall was measured and recorded. If the mouse fell off in less than 5 seconds, a second trial was performed, but no more than two trials were given per session.

Rotarod

The rotarod test was used to evaluate motor coordination (Crawley, 1999) and motor neuron degeneration (Barlow et al., 1996). A horizontal rod was rotated above a padded floor (Norflus et al., 1998). The speed of the rod was set at 24 rpm for a maximum 120 seconds. Mice were placed in the centre of the rod and latency to fall was recorded (Fig. 1B). A value of 120 seconds was recorded for mice that stayed on the rotarod for the assigned maximum duration of rotation. Rotarod was measured twice per week.

Gait length

Gait abnormalities can be detected by the de Medinaceli pawprint test (Barlow et al., 1996; Carter et al., 1999; Crawley et al., 1997; de Medinaceli et al., 1982). A dark tunnel was constructed of corrugated plastic and enclosed on all sides but the top one, with the dimensions 10 cm wide, 10 cm wide, 50 cm long. The hind paws of each mouse were dipped in Tempera non-toxic finger paint (Proart; Beaverton, OR) and it was placed on the open end of the tunnel. At the opposite end of the tunnel was the home cage of the mouse that served as incentive for the animal to pass through the tunnel. The mouse left its footprint patterns as it moved to the end of the tunnel on strips of 10 cm x 50 cm precut paper (Fig. 1C). When the mouse reached the opposite end of the tunnel it would enter its home cage and the paper strip was taken for gait length analysis. Distances between ipsilateral footprints were measured and each recorded gait length was added to calculate the average gait length for each mouse per trial.

Leg extension reflex

Normally, a mouse will extend its hind limbs away from its torso when it is lifted up by its tail (Fig. 1D). A mouse with motor neuron deficits will retract its hind limbs towards its torso (Barneoud and Curet, 1999). The original version of this test operated on a scale range from 0 to 2, 0 being no hind limbs extended and 2 being both hind limbs extended. To increase the sensitivity of this test, a 0 to 4 scale was used as shown below

Table 3

Leg extension reflex scoring

Score	Behaviour
0	Both hind limbs retracted
1	One hind limb retracted, one hind limb shaking
2	Both hind limbs shaking
3	One hind limb extended, one hind limb shaking
4	Both hind limbs extended

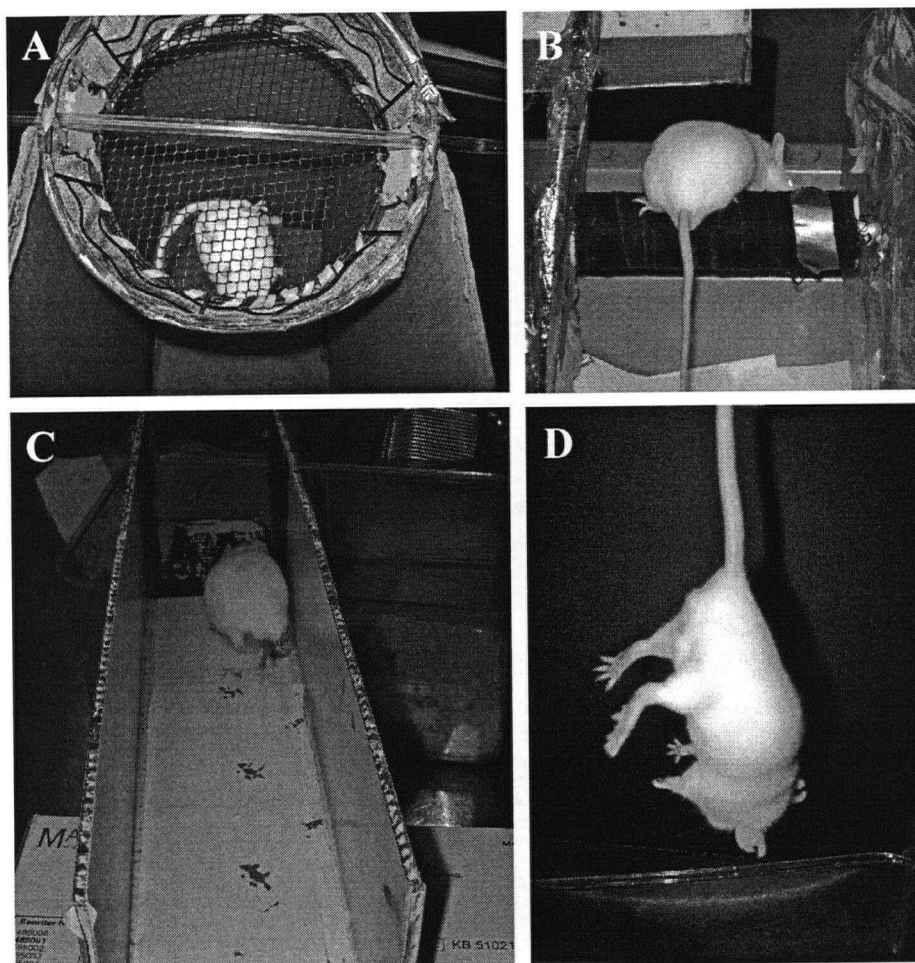


Figure 1. Motor tests. A: For the wire mesh hang test, each mouse was placed in the middle of a wire mesh grid suspended 40 cm above a padded surface, inverted and allowed to grasp the wire with all four paws. The latency to fall from the wire mesh was recorded to a maximum of 60 seconds (Crawley, 2000; Sango et al., 1996; Wallace et al., 1980). B: For the rotarod test, each mouse was placed onto a horizontal rod set at 24 rpm for a maximum 120 seconds (Crawley, 1999). C: For the gait length test, the hind paws of each mouse were painted in non-toxic finger paint and allowed to travel to the end of an enclosed tunnel (10 x 10 x 50 cm). Distances between ipsilateral paw prints left on a strip of paper were measured for gait length (de Medinaceli et al., 1982). D: For the leg extension reflex test, mice were lifted up by their tail from their home cage and the extension(s) of their hind limbs were scored (Barneoud and Curet, 1999). Normal mice would extend both hind limbs away from their torsos as shown. Scores were assigned accordingly: 0 = both hind limbs retracted, 1 = one hind limb retracted, one hind limb shaking, 2 = both hind limbs shaking, 3 = one hind limb extended, one hind limb shaking, and 4 = both hind limbs extended.

Cognitive and Emotional Tests

Water Maze

The water maze was used to evaluate spatial and reference memory, both forms of long-term memory (Morris, 1984). The water maze set-up included a pool, 1.3 m in diameter (Everts and Koolhaas, 1999), 5 radial arms, 30 cm high and a rescue platform 5 mm above water level with starting water temperature at 24°C (Fig. 2A). The mice were trained for 4 d at 3 trials/day prior to the injection regime. Mice were placed into the pool at the same start location for each trial and were allowed to explore the pool for a maximum of 60s, after which they were guided to the platform using a ruler. At 90s, the handler placed mice on the platform if they had still not reached it on their own. Training was terminated when mice consistently found the platform within 25s on 4 consecutive trials. Testing was conducted once a week for the duration of the experiment. During testing, an error was scored if the mouse fully entered an incorrect arm of the maze.

Open Field

An open field test was used to evaluate anxiety (DeFries et al., 1974). The open field arena consisted of a brightly lit open field pool, 1.3 m in diameter, 30 cm high containing mouse bedding approximately 5 cm thick (Fig. 2B). An overhead video camera was used to record mouse movement. We counted the number of squares crossed in a measured area (outside, inside and center perimeters) over a 5 minutes period. Anxiety, or fear-related behavior, is seen when the mouse remains near the edges of the arena

(thigmotaxis) rather than moving into the center of the arena (Crawley et al., 1997).

Testing was conducted once a week for the duration of the experiment.

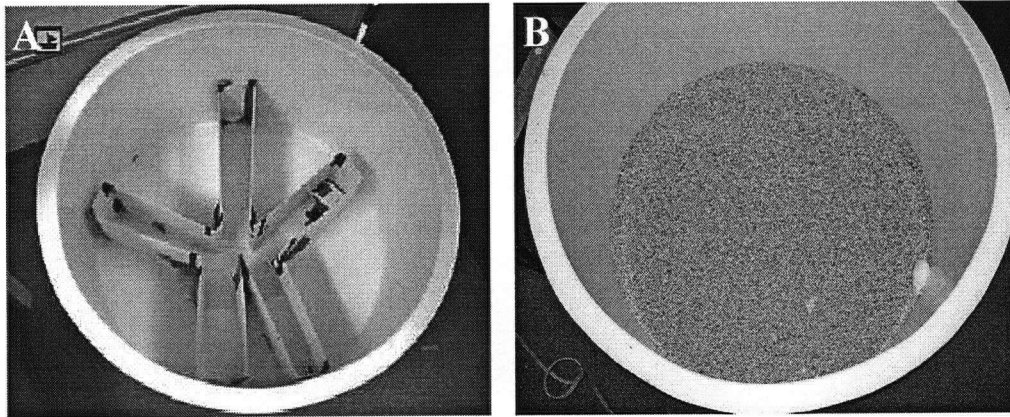


Figure 2. Cognitive and emotional tests. A: The water maze test consisted of a water pool, 1.3 m in diameter containing 5 radial arms and a rescue platform 5mm above water level. Mice were placed at the same start location for each trial and were allowed to explore the pool for a maximum of 60s, after which they were guided to the platform using a ruler. The time taken to locate the rescue platform was recorded for 3 trials per mouse to test spatial and reference memory (Morris, 1984). B: The open field test consisted of an open arena 1.3 m in diameter, 30 cm high containing mouse bedding approximately 5 cm thick. Mice were placed into the arena at the same starting point and allowed to roam freely and their movement was recorded via video for 5 minutes. The number of times a mouse crossed into any of the 3 perimeters (outside, inside and center) was recorded and analyzed. Anxiety, or fear, is typically seen with the animal reverts to the edges of the arena rather than the center (Crawley et al., 1997).

Immunohistochemistry

Perfusions, sectioning and storage

Mice were anaesthetized with an overdose of 2-bromo-2-chloro-1,1,1-trifluoroethane (halothane, Sigma), placed on soaked cotton balls in a closed chamber, and transcardially perfused using 20 mL phosphate buffered saline (PBS) and 20 mL 4% paraformaldehyde (PFA). Fixed brain and spinal cords from all mice were rapidly collected and stored in

4% PFA at 4°C for 24 hours, transferred to a 30% sucrose/PBS solution at 4°C for 24 hours and then rapidly frozen in 2-methylbutane (Fisher Scientific, Nepean, ON) on dry ice and stored at -20°C until sectioning. Spinal cords were dissected into cervical, thoracic, lumbar and sacral segments (using a spinal cord atlas, Sidman et al., 1971) before freezing with 2-methylbutane. Brains were dissected into olfactory bulbs, cortex and cerebellum. The CNS sections were cryoprotected in 30% ethylene glycol-20% glycerol-dibasic and monobasic sodium phosphate solution and kept frozen at -20°C until use. All CNS tissue blocks were mounted in Tissue-Tek optimum cutting temperature (O.C.T) frozen section medium (Sakura, Zoeterwoude, Netherlands), and then sectioned on a Bright/Hacker cryostat (Huntington, England). Brains were sectioned into 30 µm coronal slices and lumbar spinal cords were sectioned at 25 µm in the transverse plane. Sections were sequentially placed into 10 wells containing cryoprotectant working solution made using 300mL ethylene glycol and 200mL glycerol mixed with 2.73g dibasic sodium phosphate (HNa_2PO_4), 0.79g monobasic sodium phosphate ($\text{H}_2\text{NaPO}_4 \cdot 2\text{H}_2\text{O}$), and 500mL of ddH₂O for a final volume of 1L and stored at 4°C until used for histological assignment. For each immunohistological test, sections were drawn from one well and mounted onto Superfrost Plus® slides (Fisher Scientific) and allowed to dry overnight in a dessicator at room temperature before staining. Slides stained with fluorescent materials were kept in the dark at 4°C before and after microscopic viewing in order to preserve fluorescent properties.

Neuronal nuclei (NeuN) and activated caspase-3

Mouse NeuN antibody (Chemicon International; Temecula, CA, 1:300), a DNA-binding and neuron-specific nuclear protein (primarily localized in the nucleus of neurons with lighter staining in the cytoplasm) was used to identify neurons (Mullen et al., 1992; Wolf et al., 1996). Rabbit anti-activated caspase-3 antibody (Promega; Madison, WI) was used to detect cells undergoing apoptosis (Duan et al., 2003). A serial approach was used for double-fluorescence labeling due to having to use the Vector mouse on mouse (MOM) kit for NeuN. All steps were performed at room temperature unless specified otherwise. Brain slices from age-matched and experimentally naïve male mice were used as positive controls for NeuN. ApopTag® positive control slides (Chemicon) were used as positive controls for activated caspase-3. ApopTag® slides consist of 5 µm slices of rat mammary glands obtained on the fourth day after weaning when this tissue naturally undergoes apoptosis following lactation

(<http://www.chemicon.com/browse/productdetail.asp?ProductID=S7115>).

Negative control slides for NeuN were prepared with brain and lumbar spinal cord slices, negative control slides for activated caspase-3 were prepared from ApopTag® positive slides and lumbar spinal cord slices. Mounted sections were rinsed in phosphate buffered saline (PBS, Sigma) twice for 2 minutes and then placed in a coplin jar containing 10% tris-ethylene diamine tetraacetic acid (EDTA) buffer and microwaved for 10 minutes (Calbiochem International technical services, personal communication). After heating, sections were allowed to cool for 20 minutes. Slides were rinsed in PBS twice for 2 minutes and sections were then incubated in working solution of mouse on mouse

(MOM™) immunoglobulin (Ig) blocking reagent (MOM kit, Vector Laboratories, Burlingame, CA, USA) for 1 hr then rinsed with PBS twice for 2 minutes. Sections were immersed in MOM Diluent solution, prepared by adding 600 µL Protein Concentrate to 7.5 mL PBS, for 5 minutes and incubated in primary NeuN antibody for 30 minutes at room temperature and then rinsed twice in PBS for 2 minutes. NeuN negative control slides were treated identically except no antibody was added. Sections were then incubated in MOM Biotinylated Anti-Mouse Ig reagent, prepared by adding 10 µL MOM Biotinylated Anti-Mouse Ig Reagent stock to 2.5 mL MOM diluent, for 10 minutes and rinsed off with PBS twice for 2 minutes. Sections were incubated with Fluorescein Avidin DCS, prepared by adding 40 µL Fluorescein Avidin DCS stock to 2.5 mL PBS, twice for 5 minutes, then blocked with 10% normal goat serum (NGS, Invitrogen Corporation, Auckland, New Zealand) for 1 hr and rinsed twice for 2 minutes in PBS. Sections were incubated with rabbit anti-activated caspase-3 antibody (Promega; Madison, WI, 1:250 in PBS with 1% NGS) overnight in a refrigerator. Negative control slides for activated caspase-3 were treated identically except no antibody was added. PBS was then used to rinse the slides three times for 2 minutes each. Sections were then incubated in anti-rabbit AlexaFluor 546™ secondary antibody for 30 minutes at room temperature (Molecular Probes; Eugene, OR, 1:500) and rinsed off three times for 2 minutes in PBS. Sections were coverslipped using Vectashield mounting medium with fluorescent DAPI (4',6 diamidino-2-phenylindole, Vector Laboratories, Burlington, ON), sealed with clear nail polish, and allowed to air dry.

Fluoro-Jade B

Fluoro-Jade B (FJB) (Calbiochem) is an anionic fluorescein derivative that has been reported to specifically stain degenerating neurons suffering from necrotic neuronal damage (Schmued and Hopkins, 2000). It produces green iridescence with an excitation peak at 480 nm and emission peak at 525 nm. FJB is faster and more reliable than older methods (e.g. suppressed silver) for the unequivocal qualitative detection and quantitative measurement of both gross and fine scale neuronal degeneration. Positive control slides included lumbar spinal cord sections from CD-1 mice injected with kainic acid (Sigma, 10 mg/kg, i.p.) and sacrificed 4 days later (Sepkuty et al., 2002). Negative control slides consisted of lumbar spinal cord sections from experimental animals, as well as kainic acid and saline treated animals. Mounted sections were immersed in 80% absolute ethanol and 1% NaOH for 5 minutes, 70% ethanol for 2 minutes, ddH₂O for 2 minutes, 0.06% potassium permanganate for 10 minutes and ddH₂O for 2 minutes. The following steps were performed in the darkest conditions possible (room with lights turned off and slides hidden). Sections were immersed in 0.001% Fluoro-Jade B solution (0.01% stock solution made with 50 mg Fluoro-Jade B and 500mL ddH₂O, 0.004% working solution made with 4 mL of stock solution, 96 μ L acetic acid, and ddH₂O for a total volume of 100 mL) for 20 minutes and rinsed three times in ddH₂O for 1 minute per rinse. Negative control slides were immersed in an identical solution but without Fluoro-Jade B. Slides were removed from solution and allowed to dry overnight. Sections were cleared in 100% xylene three times for 2 minutes and then cover-slipped with DPX (Electron Microscopy Sciences Inc., Hatfield, PA) and allowed to air dry.

Choline acetyltransferase (ChAT)

ChAT antibody (AB144P, Chemicon International; Temecula, CA, 1:100) was used to identify cholinergic neurons in the brain and spinal cord. It is used as a specific marker for spinal motor neurons (Wetts and Vaughn, 1996; Maatkamp et al., 2004). Fluorescent immunolabeling was performed on mounted sections pretreated with 0.5% Triton X-100 in buffer for 2 x 15 minutes. Sections were then blocked in 5% NGS (normal goat serum) with 5% BSA (bovine serum albumin) for 3 hr, then incubated in goat anti-ChAT IgG antibody (in PBS with 5% NGS + 1% BSA, 1:100) overnight at 4°C. The sections were incubated for 2 hr each in rabbit anti-goat IgG antibody (1:200; DuoLuX™, Elite ABC Kit, Vector Laboratories) at room temperature and mounted with Vectashield mounting medium with fluorescent DAPI (Vector Laboratories), sealed with clear nail polish, and allowed to air dry.

Glial fibrillary acidic protein (GFAP)

GFAP is a member of the class III intermediate filament protein family and stains reactive astrocytes (which are larger in size and contain a higher number of processes than non-reactive astrocytes) following CNS injury in rodent and humans (Lee et al., 1984; Tohyama et al., 1991). Anti-Glial Fibrillary Acidic Protein Rat monoclonal antibody (345860, Calbiochem, San Diego, CA, 1:100) was used to identify astrocytes in the lumbar segment of animal spinal cord. All steps were performed at room temperature unless specified otherwise. Lumbar spinal cord sections from age-matched male mice not involved in the experiment were used as positive controls for GFAP. Lumbar spinal cord

sections incubated in buffer lacking the primary GFAP antibodies served as negative controls. Fluorescent immunolabeling was performed on slide mounted sections and pretreated in 0.5% Triton X-100 (Fisher Scientific; Fairlawn, NJ) in buffer (PBST) for 2 x 5 minutes. Sections were then blocked in 10% NGS + 1%BSA in PBST for 2 hr, followed by two rinses in PBST, 5 minutes per rinse, then incubated with primary antibody rat-anti-GFAP (in PBST with 1%NGS + 1%BSA) at 10ug/ml (1:100) in a humidified chamber at room temperature (23°C) overnight. Negative control slides were incubated using an identical solution but without the GFAP antibodies under the same conditions. Sections were then rinsed three times in PBS, 2 minutes per rinse, and then incubated for 1 hr in anti-rat Fluorescein-5-Isothiocyanate (FITC) antibody (1:200 dilution in PBS, Serotec Laboratories, Raleigh, NC, USA). This was followed by three rinses in PBS for 2 minutes each and cover-slipping using Vectashield mounting medium with fluorescent DAPI (Vector Laboratories), and finally sealed with clear nail polish and allowed to air dry.

Iba-1

Rabbit polyclonal antibody against the ionized calcium binding adapter molecule (Iba-1) (Wako, Richmond, VA, USA) was used to stain for microglia (Imai et al., 1996). For Iba-1 fluorescent immunolabeling, staining followed the same protocol used for GFAP labeling except for the following. Sections were incubated with primary rabbit-anti-Iba-1 (in PBST with 1%NGS + 1%BSA; 1:1000 dilution) overnight at 4°C. Sections were then

incubated in anti-rabbit AlexaFluor 546™ secondary antibody for 2 hours at room temperature (Molecular Probes; Eugene, OR, 1:200).

Morin (3,5,7,2',4'-Pentahydroxyflavone, BDH)

Morin (M4008-2G, Sigma) is a fluorochrome which forms a fluorescent complex with aluminum (Al) and fluoresces green (with an excitation wavelength of 420 nm) (Crapper et al., 1973; De Boni et al., 1974). The aluminum-Morin fluorescence assay was used for the visualization and detection of aluminum in lumbar spinal cord tissue. The Morin stain was used as a 0.2% solution in 85% ethyl alcohol containing 0.5% acetic acid. All mounted sections were first washed with PBS twice for 5 minutes. Sections were then pretreated for 10 minutes in a 1% aqueous solution of hydrochloric acid, rinsed in double distilled water (ddH₂O) twice for 5 minutes, and immersed in 0.2% Morin stain for 10 minutes. They were then washed in ddH₂O twice for 5 minutes, dehydrated in 70%, 90%, and 100% ethyl alcohol (EtOH), and cleared with 100% xylene. All sections were then mounted with Vectashield mounting medium with fluorescent DAPI (Vector Laboratories), sealed with clear nail polish, and allowed to air dry.

Neurofilament H

Neurofilament triplet heavy H (NFH) protein (200 kDa neurofilament protein a type of intermediate filament that occurs in both the central and peripheral nervous system and is usually neuron specific, while serving as a major element (in conjunction with other neurofilament types) of the cytoskeleton supporting the axon cytoplasm (Mendonca et al.,

2005). Abnormal accumulations of neurofilaments (NFs) in motor neurons and a down-regulation of mRNA for the NF light subunit (NF-L) are associated with ALS, but it remains unclear to what extent these NF perturbations contribute to human disease (Julien et al., 1998). Transgenic mouse models over-expressing NF proteins were found to develop motor neuron degeneration and variant alleles of the NF heavy-subunit (NF-H) gene have been found in some human ALS patients (Julien et al., 1995). Mounted sections were rinsed in PBS twice for 5 minutes and blocked with 10% NGS in PBS for 1 hour at room temperature. Following two rinses in PBST for 5 minutes, sections were incubated with primary antibody rabbit-anti-NFH (in PBS with 1% NGS) at 1:200 dilution, and incubated overnight at 4°C in a humidified chamber. Sections were then washed in PBS twice for 5 minutes and incubated in for 30 minutes in anti-rat FITC (1:200 dilution in PBS). This was followed by three rinses with PBS for 2 minutes and slides were then mounted with Vectashield mounting medium with fluorescent DAPI (Vector Laboratories), sealed with clear nail polish, and allowed to air dry.

Microscopy

Brain and spinal cord sections processed with fluorescent materials were viewed with a Zeiss Axiovert 200M (Carl Zeiss Canada Limited, Toronto, ON, Canada) microscope at 40x and 100x (under oil) magnification. DAPI (blue fluorescence) was viewed with a 359/461 nm absorption/emission filter; Alexa Fluor 546™ (red), and rabbit IgG DuoLuX™ (red) were viewed with 556,557/572,573 nm filter; FITC was viewed with a 490,494/520,525 nm filter. Brain and lumbar spinal cord sections for histology were

chosen randomly for each group. When counting using 40x magnification, two images were captured per lumbar cord section: ventral left, ventral right. 40x images were 350 x 275 μm and 100x images were 50 x 115 μm . Images were captured using AxioVision 4.3 software.

Histological Measurements

Criteria for determination and quantification of labeled cells

For quantification, only cells that were in focus and completely within the field of view were counted. To eliminate the likelihood that the same cell would be counted twice, slices for each histological experiment were drawn from one well only to ensure that sections were at least 250 μm apart. Regions of interest (ROI) for cell counts were defined using landmarks and reference points from mouse spinal cord and brain stereotaxic atlases (Sidman et al., 1971; Paxinos and Franklin K.B.J. 2001). In the spinal cord, only cells which were anterior to the central canal and deep apex where the grey and white matter meet were considered as part of the ventral horns, conversely, only cells which were posterior to the central canal and the posterior deep apex were considered as part of the dorsal horns and involved in the counting. In the brain, only cells found within the corresponding brain structures were counted. All sections were counted in a blind and unbiased manner (a code key was assigned to the animals for tracking purposes, but did not reveal the identity of treatment the animal was prescribed).

NeuN and active caspase-3

Lumbar spinal cord (n=8) and brain (n=3) sections from each mouse were examined. Five mice from each treatment group were used for assays of both lumbar spinal cord and brain. Fluorescent intensity levels of NeuN and activated caspase-3 were used to identify specific antibody labeling. Stained sections included tissue from lumbar spinal cord, primary motor cortex, the red nucleus, substantia nigra, and the dentate gyrus of the hippocampus. Cell counts included the total number of cells labeled with either NeuN, activated caspase-3, or both (double labeling) counted under a 40x objective lens.

For Fluoro-Jade B, ChAT, GFAP, and Neurofilament H I used the following procedures:

Lumbar spinal cord sections (n=8) from each mouse were captured and ROIs defined using the methods described above. Eight mice from each treatment group were used for the assay of lumbar spinal cord.

For Iba-1 and Morin I used the following procedures:

Lumbar spinal cord sections (n=4) from each mouse were captured and ROIs defined using the methods described above. Four mice from each treatment group were used for the assay of lumbar spinal cord.

Fluoro-Jade B

Counts were conducted under a 40x objective lens and included all cells positively labeled with Fluoro-Jade B in the field of view.

ChAT

Ventral root motor neurons were identified by 1) being located in the ventral horn, 2) being larger than $20 \times 20 \mu\text{m}$, and 3) labeling positive with ChAT (Stephens et al., 2006; Lago and Navarro, 2006). Only cells labeled with ChAT were included in the motor neuron counts of lumbar spinal cord. All motor neurons in the field of view were counted under a 40x objective lens.

GFAP

Counts were conducted under a 40x objective lens and included all astrocytic cells in the field of view.

Iba-1

Counts were conducted under a 40x objective lens and included all cells positively labeled with Iba-1 in the field of view.

Morin

Counts were conducted under a 40x objective lens and included all cells positively labeled with fluorescent Morin in the field of view.

Neurofilament H

Counts were conducted under a 40x objective lens and included all positively labeled with NF-H in the field of view.

Squalene Antibody Assay

Serum was collected from animals via tail bleed and sent to Tulane University Health Sciences Center for analysis where Dr. Robert Garry performed the assay. Squalene was diluted 10^{-10} -fold in distilled water, applied to nitrocellulose membranes using a cotton-tipped applicator, and allowed to air-dry. The nitrocellulose membranes were then cut into 4-mm-wide strips, placed in 20-well trays, and rinsed in wash buffer (Tris-buffered saline containing 0.3% polyoxyethylene sorbitan monolaurate and 0.005% thimerosal, pH 7.4). The strips were incubated in 2 ml blocking buffer (Tris-buffered saline containing 5% powdered instant milk, 4% goat serum, and 0.008% thimerosal, pH 7.4) for 45 minutes prior to the addition of 5 μ l of mouse serum samples (1:100 to 400 dilution) followed by a further 90 minutes incubation. All incubations and washes were carried out at room temperature on a rocking platform. The blocking buffer was then removed and the strips were washed with washing buffer (three times for 5 minutes each). After the strips were washed, 2 ml of blocking buffer containing biotin conjugated to goat anti-mouse IgG (Sigma, St Louis, Mo), diluted 1:1000, was added. After 60 minutes incubation, the strips were again washed as above, and 2 ml of blocking buffer containing avidin-conjugated horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA), diluted 1:500, was added. Following another 60 minutes incubation, the strips were

washed and 2 ml buffered saline containing 30% methanol and the substrate 0.6 mg/ml 4-chloro-1-naphthol, 0.03% hydrogen peroxide (pH 7.4) was added. The reaction was allowed to proceed for 15 minutes and was stopped by rinsing the strips in distilled water. The strips were allowed to air-dry, then qualitatively scored on a scale of 0 to 4 (see Asa et al., 2002).

Statistics

Values for each mouse on the individual tasks and in the cell counts were used to calculate mean \pm S.E.M. for each group and condition. Behavioural scores and cell counts were normalized to the mean value of controls. The means were compared using one-way ANOVA, one-way ANOVA repeated measures, and Chi-square tests (Statistica, Statsoft Inc., Tulsa, OK; GraphPad Prism, San Diego, CA).

RESULTS

Behavioural Results

Refer to Figures 3-5.

Weight

No long term significant weight differences were found between adjuvant injected mice and controls (Fig. 3).

Wire mesh hang

The greatest overall effects were seen in mice injected with aluminum hydroxide. These mice showed a progressive and significant decrease in muscular strength and endurance (50% at time of sacrifice) compared to controls (100% for all data) (Fig. 4A). Squalene injected mice showed a minor decrease in muscular strength that did not achieve significance. The aluminum hydroxide and squalene (combined) group did not show any statistically significant differences in muscle strength and endurance.

Rotarod

Rotarod performance was found to be similar between adjuvant injected and control mice (Fig. 4B).

Gait length

No differences between adjuvant injected and control mice were found for gait length (Fig. 4C).

Leg extension

Performance on the leg extension test was found to be similar between adjuvant injected mice and controls (Fig. 4D).

Water Maze

Assessment of cognitive performance on the water maze showed that mice injected with aluminum hydroxide (1.2 errors) or squalene (0.9 errors) showed an increase in the number of errors after week 20, but these differences did not reach statistical significance. Mice injected with both adjuvants had significant late stage, long-term memory deficits with an increase in the number of errors after week 20 (4.3 errors) compared to controls (0.2 errors) (Fig. 5A).

Open Field

Aluminum injected mice showed a significant increase in anxiety levels at week 14 (138%) as measured by the longer time spent in the outer perimeter during the open field tests (Fig 5B). Post week 14, the aluminum group continued to show increased levels of anxiety compared to controls but these values did not reach statistical significance ($P=0.018$ at week 24). The squalene group also showed a small increase in anxiety after

week 20 but these results did not achieve statistical significance. There was no difference in anxiety levels between the combined group and controls.

Histological Results

Refer to Figures 6-12.

NeuN and activated caspase-3

Mice injected with PBS showed little or no activated caspase-3 labeling in ventral lumbar spinal cord (Fig 6C, E, G) or any other CNS region. In contrast, mice injected with aluminum hydroxide showed a significant 255% increase in activated caspase-3 labeling alone and a significant 233% increase in double labeling with NeuN (Fig. 6D, F, H-J; 7A). Activated caspase-3 was also increased in the squalene group as well as the combined aluminum and squalene group, but quantified cell counts did not reach statistical significance.

In the brain, quantitative analysis of NeuN labeling showed comparable numbers of labeled neurons in all treatment groups (Fig 7B-E). Mice injected with aluminum hydroxide showed a significant increase in activated caspase-3 labeling (192%) and activated caspase-3/ NeuN double labeling (185%) in the primary motor cortex compared to controls (Fig. 7B). The squalene and combined group showed small increases in activated caspase-3 and activated caspase-3/ NeuN double labeling but these did not reach statistical significance. Cell counts performed in the red nucleus show increased activated caspase-3 and double labeling in both aluminum groups, but these results were

not significant (Fig. 7C). Analysis of the substantia nigra region did not reveal any differences in labeling between groups (Fig 7D). In the hippocampus, cell counts conducted on the polymorphic layer of the dentate gyrus (DG) showed an increase in double labeling for squalene and combined groups but it did not reach statistical significance (Fig. 7E).

Fluoro-Jade B

Positive control slides included lumbar spinal cord sections from CD-1 mice injected with kainic acid and revealed Fluoro-Jade B labeling (Fig 8A). Regarding control and experimental groups, there was no significant labeling with Fluoro-Jade B in the lumbar spinal cord of control (Fig. 8B), aluminum (Fig. 8C), squalene or aluminum and squalene treated animals and thus no differences were found between the groups.

ChAT

Aluminum injected mice showed decreased labeling and cell shrinkage among motor neurons as well as significant reduction in motor neuron number (35%) compared to controls (Fig. 9A, B, C). The squalene and combined group also showed a reduction in motor neuron number that did not achieve statistical significance.

GFAP

The aluminum injected group showed a highly significant increase in the expression of GFAP positive astrocytes (350%) greater than controls (Fig 10A-D). Animals treated

with squalene or aluminum with squalene showed small increases in the number of astrocytes present when compared to controls but these differences were not statistically significant.

Iba-1

Highly significant increases in the expression of Iba-1 positive microglia were observed in groups injected with aluminum hydroxide (211%) and both aluminum hydroxide and squalene (233%) greater than controls (Fig 11A-C). Animals treated with squalene alone showed a small increase in the number of microglia present when compared to controls but this difference was not statistically significant.

Morin

Only mice injected with aluminum hydroxide showed significantly increased Morin labeling of cells in lumbar spinal cord compared to squalene injected mice or controls (Fig. 12A-E). Animals injected with squalene alone or PBS (controls) did not exhibit Morin fluorescence.

Neurofilament H

No differences were found between the adjuvant injected and control groups for the presence of NF-H labeling or aggregation.

Squalene Antibody Assay

Two of ten control animals showed the presence of squalene antibodies (SA) in the first serum specimen taken at 4 weeks (2 weeks post second injection). A larger number of animals, 4/10, injected with squalene possessed detectable levels of SA at this time point, however this difference was not statistically significant (Chi-square test: $F=4.011$, 3 ($p=0.2603$)). 3/11 animals injected with aluminum hydroxide and 1/10 injected with both adjuvants also showed increased SA. The presence of SA was generally stable over time in individual animals tested. However, one animal that had been injected with both adjuvants developed SA at a later time point (24 weeks).

Non-CNS features

In addition to behavioural changes and CNS pathology, various physiological changes were observed. Hair loss at the injection site (0.5 cm to 1.0cm diameter region around the injections site) was common to all adjuvant treated groups: 2/10 from the aluminum hydroxide group, 4/10 from the squalene group, and 3/10 mice from the combined group. No control animals developed hair loss in the injection area. Four of the ten mice injected with both adjuvants developed an allergic skin reaction (dermatitis; inflammation of the skin characterized by itchiness and redness with scaling) showing in a 0.5 cm diameter region around the injection site.

DISCUSSION

Although several animal studies using the anthrax vaccine have been published (Ivins et al., 1995; Fellows et al., 2001; Williamson et al., 2005), none of these experiments examined neurological outcomes or behavioural side effects. The present results show that anthrax vaccine adjuvants mimicking a minimal AVA administration regime (2 injections, Nass, M.; personal communication) resulted in some behavioural deficits and neuropathological outcomes post injection.

Interpretation of Pathological, Behavioural, and Blood Results

Pathological Effects

Injection of aluminum hydroxide induced significant motor neuron loss in the lumbar spinal cord and increased the presence of apoptotic neurons in various motor regions of CNS in male mice. The presence of active caspase-3 labeling in cells not labeled with NeuN suggests that some non-neural cells also undergo apoptosis under these conditions. In aluminum treated animals, several neurons appeared as 'unhealthy' or 'sick'. Visual inspection revealed structural irregularities such as folding of the cell itself and vacuolization of the cytoplasm, which could be an indication of a pre-apoptotic condition. Previous studies have demonstrated that autophagic vacuoles can precede apoptotic cell death (Gonzalez-Polo et al., 2005). Activated caspase-3 labeling suggests that some neurons are dying by apoptosis rather than necrosis. However, activated caspase labeling

alone does not imply lack of cell death by necrosis. I performed a FJB assay to further investigate for evidence of neuronal necrosis in the CNS, but the lack of positive FJB labeling suggested no demonstrative necrotic cell death. In addition to positive caspase labeling and the absence of FJB labeling, evidence for apoptotic processes included the deletion of single cells (not cell groups), shrinkage of somata (this morphology was also seen in motor neurons after ChAT labeling), membrane folding (but without loss of integrity), and definite compaction of chromatin into uniformly dense masses.

In addition to the spinal cord, I also examined other brain structures involved in motor function. NeuN and activated caspase-3 immunohistology was performed on the primary motor cortex, the red nucleus, substantia nigra, and hippocampus since these areas are affected in the human motor diseases ALS and Parkinson's (Sasaki et al., 1992; Eisen and Weber, 2001; Tsuchiya et al., 2002). Treatment with aluminum hydroxide showed the greatest evidence for neuronal damage in the brain. In this group, the primary motor cortex showed significantly increased activated caspase-3 labeling compared to controls and there were small indications (non significant, but a trend) of activated caspase-3 labeling in the red nucleus. The combination of both adjuvants showed a significant long-term memory deficit but no significant indications of neuronal apoptosis in the red nucleus or DG region of the hippocampus. These findings demonstrate apoptotic processes occurring within the brain in addition to spinal cord. Unfortunately, at present, no extensive post mortem studies have been published on patients with GWI and the involvement of these areas and other brain regions is not known. Morin staining revealed

the presence of aluminum in the cell body and/or nucleus of some neurons in both aluminum treated groups with no indications of aluminum in squalene or control groups, suggesting involvement of aluminum in neurotoxicity. These results are consistent with a potential role for aluminum in motor neuron death in ALS. In CNS areas tested to date (spinal cord), reactive astrocytes were present in significant numbers, indicating astrogliosis, and significant microglial proliferation was present. Previous studies have shown the increased presence of reactive astrocytes in human ALS (Nagy et al., 1994; O'Reilly et al., 1995) and animal models of the disease (Levine et al., 1999; Barbeito et al., 2004). There was no clear evidence for significant neuropathology with squalene treatment in either brain or spinal cord.

Behavioural Effects

Aluminum hydroxide induced behavioural abnormalities in motor and cognitive function. The wire mesh hang test showed the greatest deficit in the aluminum treated group, however I cannot ascertain whether this deficit reflects primary muscle involvement (i.e., alterations at the neuromuscular junction) or an alteration in joint structure. Motor function loss that can arise due to the pathology previously described, but the observed deficit cannot be solely motor neuron loss since there is no significant leg extension loss. The lack of leg extension deficits seen in the aluminum group can also be explained by illustrating a motor neuron count versus performance score correlation threshold (see Fig. 13). Previous findings from our group have shown that mice can have up to approximately 40% motor neuron loss and still not show any significantly reduced leg

extension scores (Wilson et al., unpublished). Thus, while motor neuron loss may be involved, other events are likely occurring. In particular, there could exist complications at the neuromuscular junction (as seen in mSOD mice; Fisher et al., 2003) that result in the wire hang deficits, but not necessarily enough motor neuron death to give loss of the leg extension reflex. This, in turn, suggests that the muscle and endplate are early targets for toxins that may generate ALS-like motor neuron loss. These observations could explain the lack of negative outcomes not measurable on this task as the aluminum treated group only demonstrated approximately 35% motor neuron loss in the lumbar spinal cord. The phenomenon of motor neuron loss with no clinical symptoms is consistent with findings from a separate mouse study (Kong and Xu, 1998) and limited spread of motoneuronal signs found during diagnosis of ALS in human patients (Zoccolella et al., 2006a; Zoccolella et al., 2006b).

The squalene adjuvant alone produced a small change in anxiety testing, but the differences in the cell counts of this group with respect to controls were not significant in any CNS region. Thus, while squalene does not appear to have the same overall impact as aluminum at sacrifice, the change in cognitive function may suggest that possible longer-term squalene effects should be examined in future studies. In this study, the dose of squalene was relatively low compared to that used in the MF59 formulation (and possibly human AVA) (2% versus 5% concentration), and the effects could be a dose dependent, especially since deleterious effects have been observed at higher doses (see Introduction).

Blood Analysis

In regard to our SA assays, we were able to detect antibodies in 40% of the mice injected with squalene. This outcome was the highest incidence level of all treatment groups, however, the other groups, including controls, showed some SA positive mice. Previous studies have suggested that naturally occurring antibodies to squalene develop in mice, as well as humans, during the aging process (Matyas et al., 2004). BALB/c, B10.Br and C57BL/6 mice showed SA in approximately 12% of animals, similar to our control and aluminum hydroxide injected CD-1 mice. The relatively low incidence of SA in squalene injected mice may reflect a transient antibody production. Future experiments with more specific antibodies may resolve this issue.

Neurotoxic Outcomes and Plausible Mechanisms

Aluminum

Various studies have clearly demonstrated that aluminum, in both oral and injected forms, can be neurotoxic (Crapper et al., 1973; Banks and Kastin, 1989; Joshi, 1990; Kawahara et al., 2001). Aluminum has been widely proposed as a factor in neurodegenerative diseases based on its demonstrated neurotoxic properties and its association with degenerating neurons in specific CNS areas (Perl et al., 1982; Perl and Pendlebury, 1986; Rao et al., 1998; Savory and Garruto, 1998). In neurodegenerative disease, aluminum has been linked to the accumulation of tau protein and amyloid-beta protein in experimental animals and observed to induce neuronal apoptosis in vivo as

well as in vitro (Kawahara, 2005). Also, aluminum injected animals show severe anterograde degeneration of cholinergic terminals in cortex and hippocampus (Platt et al., 2001). Aluminum in its adjuvant form can access the CNS (Wen and Wisniewski, 1985, 1985; Redhead et al., 1992; Sahin et al., 1994), however, oral administration of aluminum hydroxide gels does not appear to be neurotoxic in humans (Rosati et al., 1980). In aluminum adjuvants, the route of exposure appears to be a key factor that determines its neurotoxic effect. Potential toxic mechanisms of action for aluminum may include enhancement of inflammation (i.e. microgliosis) and the interference with cholinergic projections (Platt et al., 2001), reduced glucose utilization (Joshi, 1990), defective phosphorylation-dephosphorylation reactions (Cordeiro et al., 2003), altered rate of transmembrane diffusion and selective changes in saturable transport systems in the blood brain barrier (BBB) (Kaya et al., 2003), and oxidative damage on cellular biological processes by inhibiting glutathione regeneration (Murakami and Yoshino, 2004).

I speculate that the observed neurotoxic effects of aluminum hydroxide in this study arose by both 'direct' and 'indirect' pathways. Direct toxicity refers to the physical presence (or close proximity) of aluminum and its effect on initiating cell death. In terms of cell pathogenesis, this typically includes localization of aluminum within the cell body and its surrounding environment. This is largely characterized by accumulation of aluminum via cell uptake (i.e. passive diffusion) into the cytoplasm where the metal could cause alterations in glutaminase and glutamine synthetase (via increased

intracellular glutamine levels, decreased intracellular glutamate levels, and increased conversion of glutamate to glutamine and the release of the latter into the extracellular space) and easily alter the availability of neurotransmitter glutamate (Zielke et al., 1993). Within the cell, aluminum could also produce accumulations of neurofilaments (NF), e.g., neurofibrillary tangles (NFT), in neuronal cell bodies and proximal axonal segments that ultimately impair NF transport (Bizzi et al., 1984). Outside the cell, aluminum may affect the neuron by altering end plate structure. For example, aluminum has been shown to decrease the thickness of post-synaptic density, increase the width of the synaptic cleft, and increase numbers of flat synapses (Jing et al., 2004). Extracellularly, aluminum could also block voltage activated calcium channels (Busselberg et al., 1993), augment the activity of acetylcholinesterase (Zatta et al., 2002), or interfere with synaptic transmission by merely accumulating in the synaptic cleft (Banin and Meiri, 1987). In addition, exogenous compounds such as citrate may be a chelator of aluminum for its cellular uptake (Bittar et al., 1992). This chelating mechanism is known to impair astrocyte metabolism while aggravating the accumulation of aluminum. Aluminum can also induce apoptosis in astrocytes (Aremu and Meshitsuka, 2005). Thus, loss of astrocytic regulatory and supportive roles in the central nervous system (CNS) may also be responsible for neuropathology observed in this study. Morin staining indicated the presence of aluminum within the cell body of several neurons and it is likely this internalization is mostly responsible for the cell death observed; however, cell death via an 'indirect' pathway may also occur.

Indirect toxicity refers to the triggering of a neuropathological cascade by aluminum (or another agent), where localization of the agent does not occur near the targeted cell but is derived elsewhere. During indirect toxicity, degenerating cells have no physical contact with aluminum, and the metal is likely situated or stored elsewhere (i.e., the lymphatic system after subcutaneous injection). Aluminum can deregulate pro-inflammatory cytokines, which can damage cells (Johnson and Sharma, 2003), cause the release of glutamate and gamma-amino butyrate (GABA), and modify enzyme activity, which can lead to neuropathology (Nayak and Chatterjee, 2001).

Aluminum and squalene

In addition to direct toxic actions on the CNS, aluminum and squalene might act indirectly by stimulation of a generalized immune response. This is, in fact, what adjuvants are placed in vaccines to do in the first place. Adjuvant neurotoxicity may be the result of an imbalanced immune response. Rook and Zumla (1997) hypothesize that multiple Th2 (T helper cell type 2)-inducing vaccinations, stressful circumstances, and the method of vaccine administration (oral vs. subcutaneous vs. intramuscularly) could lead to a shift from Th1 (T helper cell type 1; cell mediated immunity) to Th2 (T helper cell type ; humoral immunity) immunity (Rook and Zumla, 1997; Rook and Zumla, 1998). Both aluminum hydroxide and squalene have previously been shown to stimulate a Th2-cytokine response (Valensi et al., 1994; Brewer et al., 1999). A recent study comparing inbred and outbred mouse strains injected with recombinant protective antigen (AVA) vaccine and challenged with *Bacillus anthracis*, found that both mouse strains

displayed a predominantly Th2 biased immune response (Flick-Smith et al., 2005). This type of Th1 to Th2 shift could stimulate autoimmune processes that target neurons. While a plausible mechanism, a recent study of blood samples from Gulf War veterans, showed evidence for Th1 immune activation (Skowera et al., 2004). Additionally, some studies suggest a potential role for autoimmune mechanisms in the destruction and loss of motor neurons in ALS (Appel et al., 1991; Appel et al., 1994ba). In human ALS, IgG selectively interacts with calcium channels and alters channel function at the neuromuscular junction (Appel et al., 1994ab; Smith et al., 1996). The chemical interactions between adjuvants are not well understood. In this study, the mixing of aluminum hydroxide with squalene prior to injection could cause physical or chemical interactions (that are not presently known) between the two compounds, thereby hindering the overall effect of one another. This could explain the lack of pathology and behavioural deficits observed in the combined group. Clearly, there is much debate concerning adjuvants and their autoimmune processes and further investigation into the driving mechanisms of these adjuvants (and other adjuvants in general) is required in order to draw any firm conclusions.

Squalene

Squalene has been shown to induce antibodies associated with lupus (Sato et al., 2003) and to trigger chronic T-cell mediated rheumatoid arthritis (Carlson et al., 2000). One study using MF59, a squalene adjuvant, suggests that it interacts with antigen presenting cells at the site of injection and then moves to the draining lymph nodes where it

increases the efficiency of antigen presentation to T cells (Dupuis et al., 1998). Another study using this vaccine proposes that lymph node-resident dendritic cells can acquire the antigen and MF59 after intramuscular immunization by uptake of apoptotic macrophages (Dupuis et al., 2001). The actions of squalene in the CNS have not been extensively investigated, but some studies using very high concentrations have demonstrated swelling of astrocytic processes (Gajkowska et al., 1999).

Interactions of various stressors, including adjuvants, may be complex and do not have to be necessarily synergistic. For example, in the present study, the combination of aluminum hydroxide and squalene seemed to have less effect on motor behavior and anxiety than either aluminum hydroxide or squalene alone. The possibility of competing effects on immune response cannot be discounted and deserves further investigation.

Adjuvants Not the Only Suspect in GWI

While I have demonstrated significant behavioural and neuropathological outcomes with aluminum hydroxide and some additionally significant outcomes to the combination of adjuvants, it is important to recognize that these were achieved under *minimal* conditions. Table 1 shows a summary of human ALS and GWI symptoms compared with outcomes observed in aluminum-injected mice. The likelihood that a synergistic effect exists between adjuvants and other variables such as stress, multiple vaccinations, and environmental toxic exposure is another possibility that cannot be ruled out. A recent

study examining some of these combinations showed that stress, vaccination, and pyridostigmine bromide, a carbamate anticholinesterase (AChE) inhibitor, may synergistically act on multiples stress-activated kinases in the brain to cause neurological impairments in GWI (Wang et al., 2005). In addition, genetic background may play a crucial role. Recent studies have identified lower levels of serum paraoxonase (PON1), a enzyme that helps detoxify organophosphates, as well as reduced PON1 activity in Gulf War veterans compared with military control groups (Mackness et al., 2000; Hotopf et al., 2003). In regard to this last point, gene-toxin interactions remain a largely unexplored area in GWI and neurological disease in general.

While this study revealed deleterious effects of the aluminum adjuvant used in the anthrax vaccine, it did not investigate the whole vaccine preparation itself. Thus, any other possible interactions between the sole adjuvant versus the whole vaccine formulation must be taken into consideration. This study was only able to produce some of the symptoms seen in GWI and not the wide spectrum that is exhibited in GWI patients, nor all the symptoms that parallel with ALS (such as limb or tongue fasciculations and advanced impairment of extremities). Nonetheless, this study does suggest possible involvement of AVA adjuvants in inducing rather than developing some symptoms of ALS-GWI. Based on the findings, and other investigations on Gulf War veterans, I propose that GWI may be a cumulative result of several factors including the synergistic effects between multiple environmental agents, genetic predisposition and immense psychological stress from wartime service. As previously mentioned, evidence

for the efficacy of the anthrax vaccine remains unproven (Nass, 2002) and largely limited to acute animal studies (Fellows et al., 2001), while chronic side effects have not been investigated in sufficient detail. Further studies must be conducted to clarify our understanding of adjuvants and vaccines, their underlying mechanisms and their potential role in the development of Gulf War-related illnesses.

Future Studies

Although this study produced some novel findings, it was primarily exploratory in nature and designed to begin the investigation on the behavioural and neurological impact of these adjuvants in an animal model. Follow-up studies need to more specific and should perform time point analysis in order to investigate when neuronal death first occurs, whether apoptotic processes are persistent, and equally important, whether the pathological changes are occurring in tandem with observed neurological symptoms. In addition, a wider range of studies need to examine all environmental exposures incurred by Gulf War soldiers, plausible synergistic interactions of these agents, mechanisms of their toxicity and the susceptibility of certain populations based on genetic background.

GWV continues to plague veterans of the Gulf War and may be developing in troops deployed in the Iraq war. Also, the number of surviving patients with known ALS-GWV is quickly diminishing along with the opportunity to study them. Future studies should examine the pathology of patients who suffered with GWV to ascertain the pathological

similarities and differences of these patients compared to victims of other neurological diseases. Currently, little is known about GWI and its origin because few animal and human studies were performed and previous attempts to collect and chronicle data (such as records of vaccination) were inadequate. If we are to understand the causes of GWI, we must investigate all possible variables that could be potentially involved and perform the necessary scientific experiments. The results of these efforts will help build a database of knowledge may be able to provide clearer diagnoses or even treatment for future soldiers with war-related illnesses.

Implications for Future Use of the Anthrax Vaccine

Gulf War veterans have an illness that may be caused by the AVA or its adjuvants. The current DOD immunization schedule requires a higher number of injections (6) than used in 1990-1991. The majority of those vaccinated with the AVA vaccine to date have been service personnel. The current war in the Middle East has deployed the most troops since 1991 by the U.S. and Great Britain. Despite current initiatives by the U.S. government to keep accurate medical records and institute stronger preventative measures for military service personnel currently in theatre, there is a strong likelihood that we will witness the emergence of a second possible GWI variant, termed "Gulf War syndrome II", among the currently deployed military population in future years (Enserink, 2003). As serious as this may be for the potential for adjuvant-associated complications in this population, legislation already passed by U.S. Congress mandate similar vaccination regimes for the

civilian population as well (e.g., the Biodefense and Pandemic Vaccine and Drug Development Act of 2005). If a significant fraction of the military and civilians vaccinated were to develop neurological complications, the impact on U.S. society could be profound.

In addition, the continued use of aluminum adjuvants in various vaccines (i.e., Hepatitis A and B, DPT, etc.) for the general public may have even more widespread health implications. Despite two decades aimed at formulating alternative vaccines to overcome problems of efficacy, safety and supply, such an alternative is at least five years away (or longer), and so the present status is to accept the current versions of the vaccine or choose to not vaccinate at all (Turnbull, 2000). Until vaccine safety can be comprehensively demonstrated by controlled long-term studies that examine the impact on the nervous system in detail, many of those already vaccinated as well as those currently receiving injections may be at risk in the future. Whether the risk of protection from a dreaded disease outweighs the risk of toxicity is a question that demands our urgent attention.

FIGURES AND TABLES

Figure 3

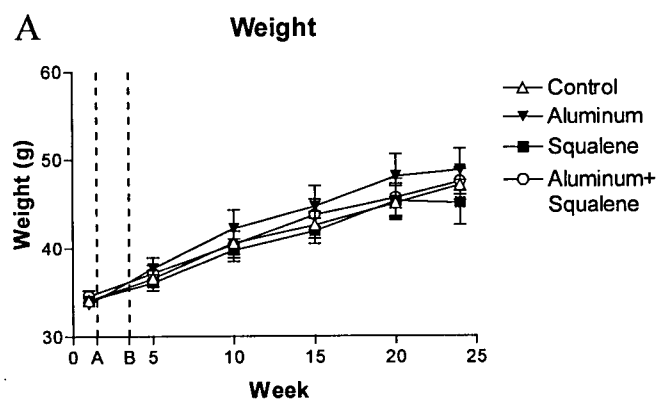


Figure 3. Recorded weight of animals. A: Weight of each animal was recorded once per week. Graph shows a linear relationship of normal weight gain with age increase and no significant differences were observed between the groups. A=1st injection, B=2nd injection.

Figure 4

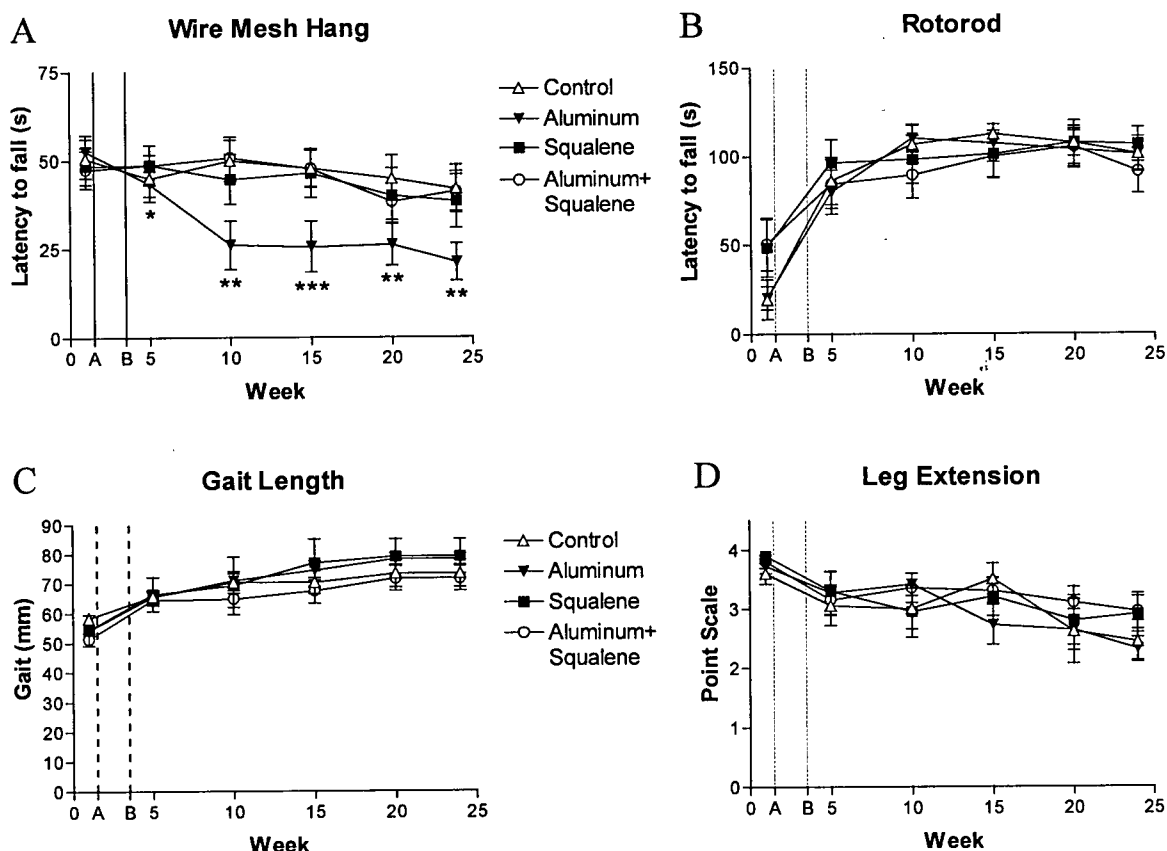


Figure 4. Effects of adjuvants on motor behaviour. A: Wire mesh hang test. Mice injected with aluminum hydroxide showed a significant decrease in muscular strength and endurance (50%) compared to controls (100%). Mice injected with squalene or both adjuvants did not show a significant decrease in muscular strength. Repeated measures ANOVA: Group: $F_{3,36}=1.86$ ($p=0.15$); Trial: $F_{5,180}=7.26$ ($p<0.001$); Interaction: $F_{15,180}=2.02$ ($p=0.02$). B-D: There were no significant differences in performance between all groups on the rotorod, gait length and leg extension tests. A=1st injection, B=2nd injection. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$, one-way ANOVA.

Figure 5

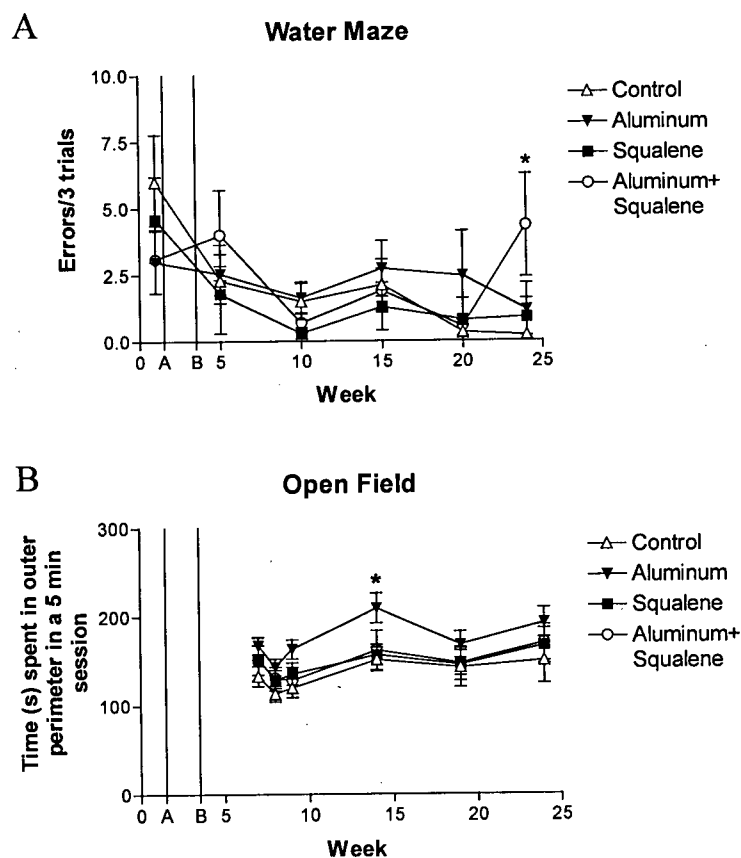
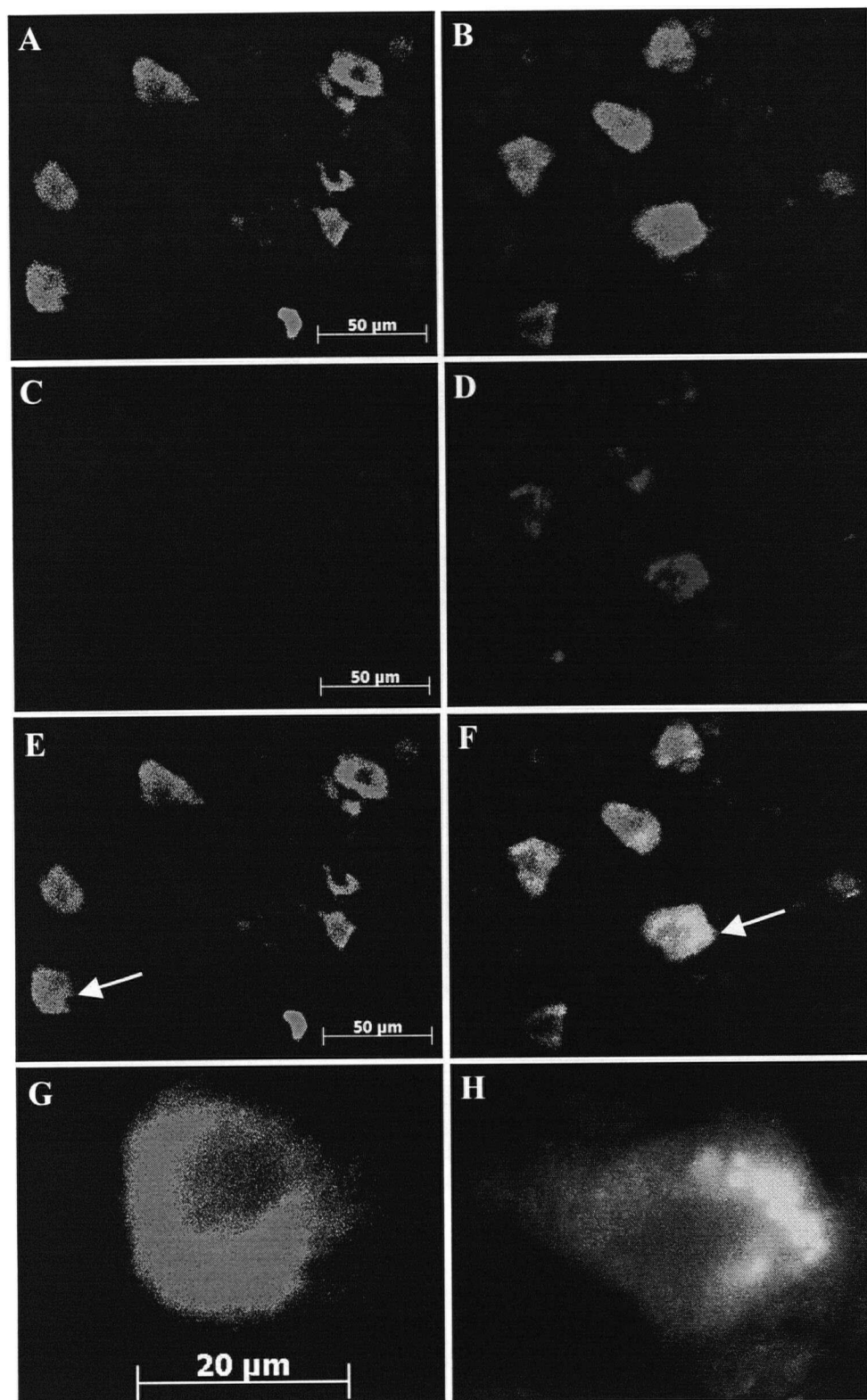


Figure 5. Effects of adjuvants on cognitive and emotional behaviour. A: The radial arm water maze (5 arms). Mice injected with both adjuvants showed a significant increase in errors after week 20 (4.3 errors) while controls achieved 0.2 errors. Mice injected with aluminum hydroxide (1.2 errors) or squalene (0.9 errors) showed increased errors after week 20 but these values did not reach statistical significance. Repeated measures ANOVA: Group: $F_{3,34}=0.36$ ($p=0.78$); Trial: $F_{5,170}=4.78$ ($p<0.0001$); Interaction: $F_{5,170}=1.22$ ($p=0.26$). B: Open field tests (during weeks 7-24). Mice injected with aluminum hydroxide show a significant increase in anxiety (138%) compared to controls. Mice injected with squalene or both adjuvants did not show any significant effect. Repeated measures ANOVA: Group: $F_{3,34}=1.63$ ($p=0.20$); Trial: $F_{5,170}=10.64$ ($p<0.0001$); Interaction: $F_{15,170}=0.45$ ($p=0.96$). A=1st injection, B=2nd injection. *, $p<0.05$, one-way ANOVA.

Figure 6



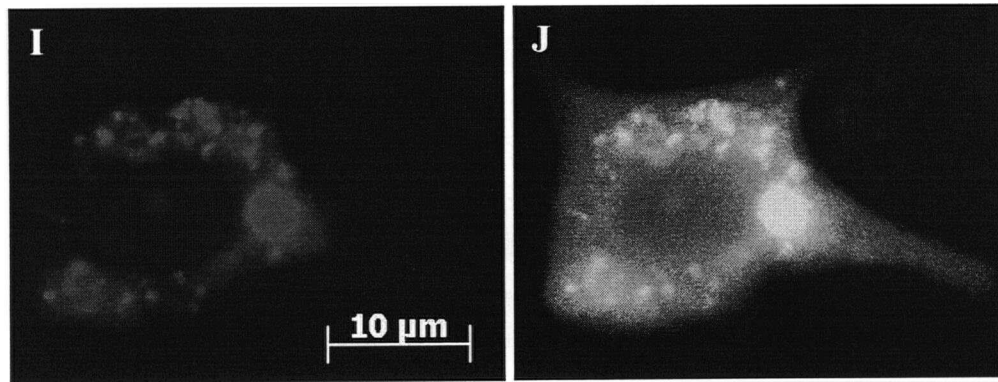


Figure 6. NeuN and activated caspase-3 fluorescent labeling in ventral horn of lumbar spinal cord. A-B: NeuN labeling in control and aluminum hydroxide injected mouse lumbar spinal cord sections, respective. C-D: Control and aluminum hydroxide mouse lumbar spinal cord sections labeled with caspase-3. E-F: Merge of NeuN and caspase. Magnification: 40x A-F. White arrow indicates neuron enlarged in G-H. G-H: Enlargement of neurons E-F at 100x magnification. I-J: Enlargement of another activated caspase-3 positive motor neuron at 100x magnification. J: Merged image of activated caspase-3 and NeuN. A-F: Scale bar = 50 µm. G, H: Scale bar = 20µm. I, J: Scale bar = 10µm.

Figure 7

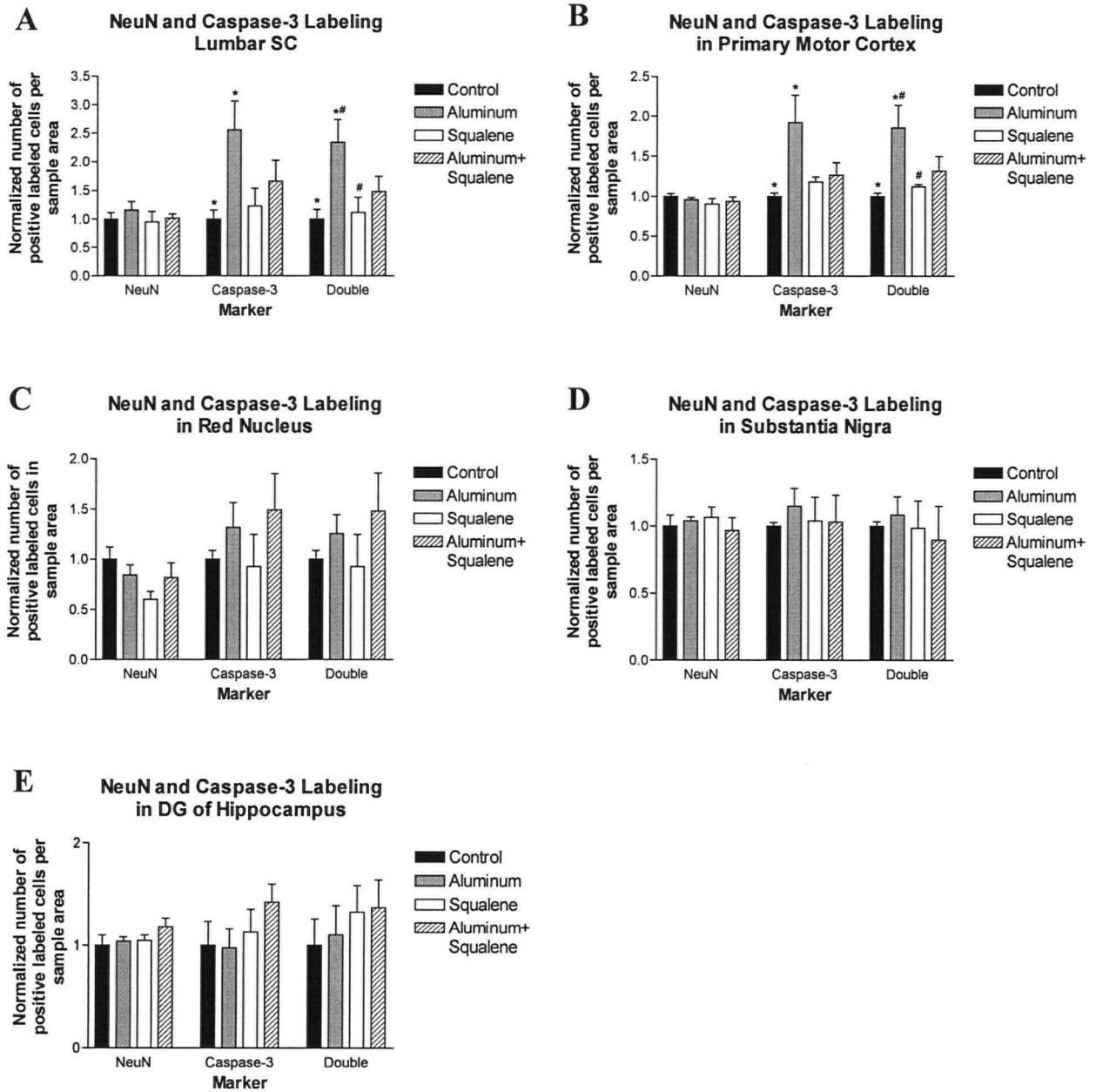


Figure 7. Cell counts for NeuN and activated caspase-3 labeling in ventral horn of lumbar spinal cord. A: NeuN counts between groups (n=32, 8 per group) show no significant differences indicating similar numbers of neuronal cells labeled in all groups. Activated caspase-3 marker shows significantly increased positive capsase-3 labeling (255%) in mice injected with aluminum hydroxide compared to controls. NeuN and activated caspase-3 double labeling show significantly increased apoptotic neuronal cells (233%) in mice injected with aluminum hydroxide compared to control and squalene injected groups. B: NeuN counts (n=20, 5 per group) in the primary motor cortex show no significant difference between groups. Animals injected with aluminum hydroxide show a significant increase in activated caspase-3 (192%) and double labeling (185%) in primary motor cortex compared to controls. Aluminum hydroxide injected mice showed a significant increase (165%) in double labeling when compared to squalene-injected mice. C: Cell counts (n=20, 5 per group) performed in the red nucleus show a non significant increase in activated caspase-3 and double labeling in both aluminum groups compared to controls. D: SNpc: There was no significant difference in cell counts (n=20, 5 per group) of NeuN and activated caspase-3 labeling between groups in the substantia nigra region. E: Hippocampal cell counts (n=20, 5 per group) performed on the polymorphic layer of the dentate gyrus show increased activated caspase-3 and double labeling in the squalene group, while the combined group showed the greatest activated caspase-3 and double labeling. These results were not statistically significant. Histograms show means \pm S.E.M * $p < 0.05$ aluminum versus control mice, # $p < 0.05$ aluminum versus squalene mice; using one-way ANOVA.

Figure 8

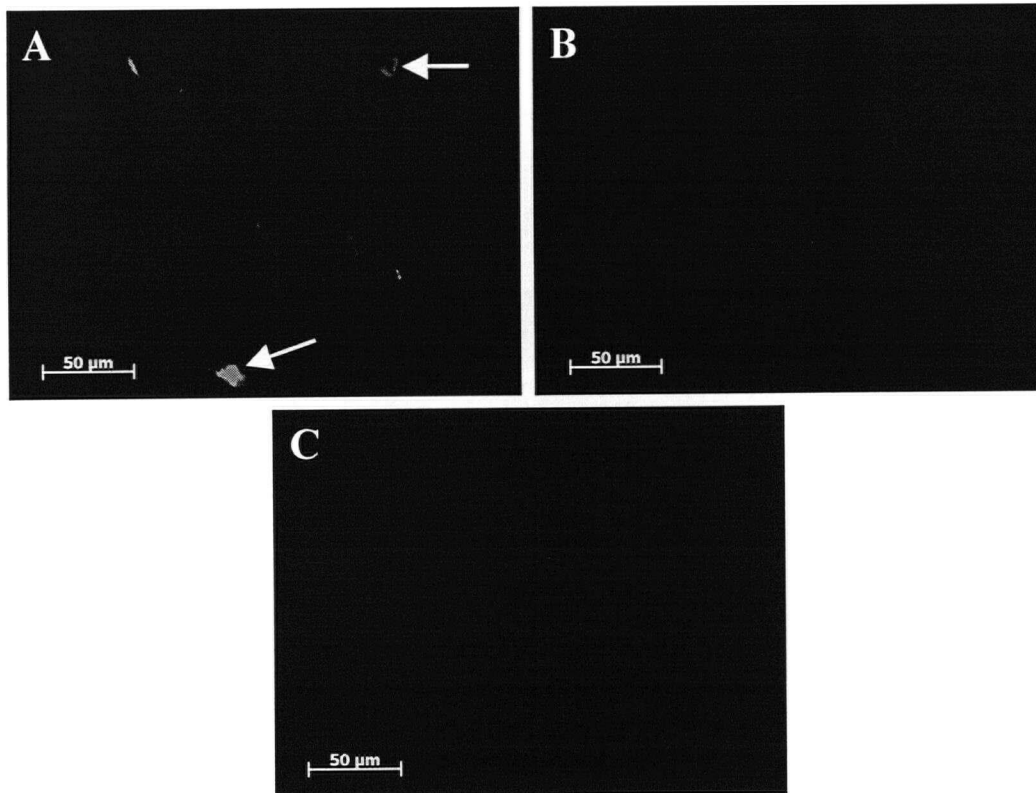


Figure 8. Fluoro-Jade B immunoreactivity in ventral horn of lumbar spinal cord. A: Positive Fluoro-Jade B labeling in CD-1 mouse injected with kainic acid indicating necrotic neuronal damage (positive control). B, C: Control (B) and aluminum treated (C) mice show no positive labeling for Fluoro-Jade and no indications of neuronal necrosis. A-C: 40x magnification. Scale bar = 50 µm.

Figure 9

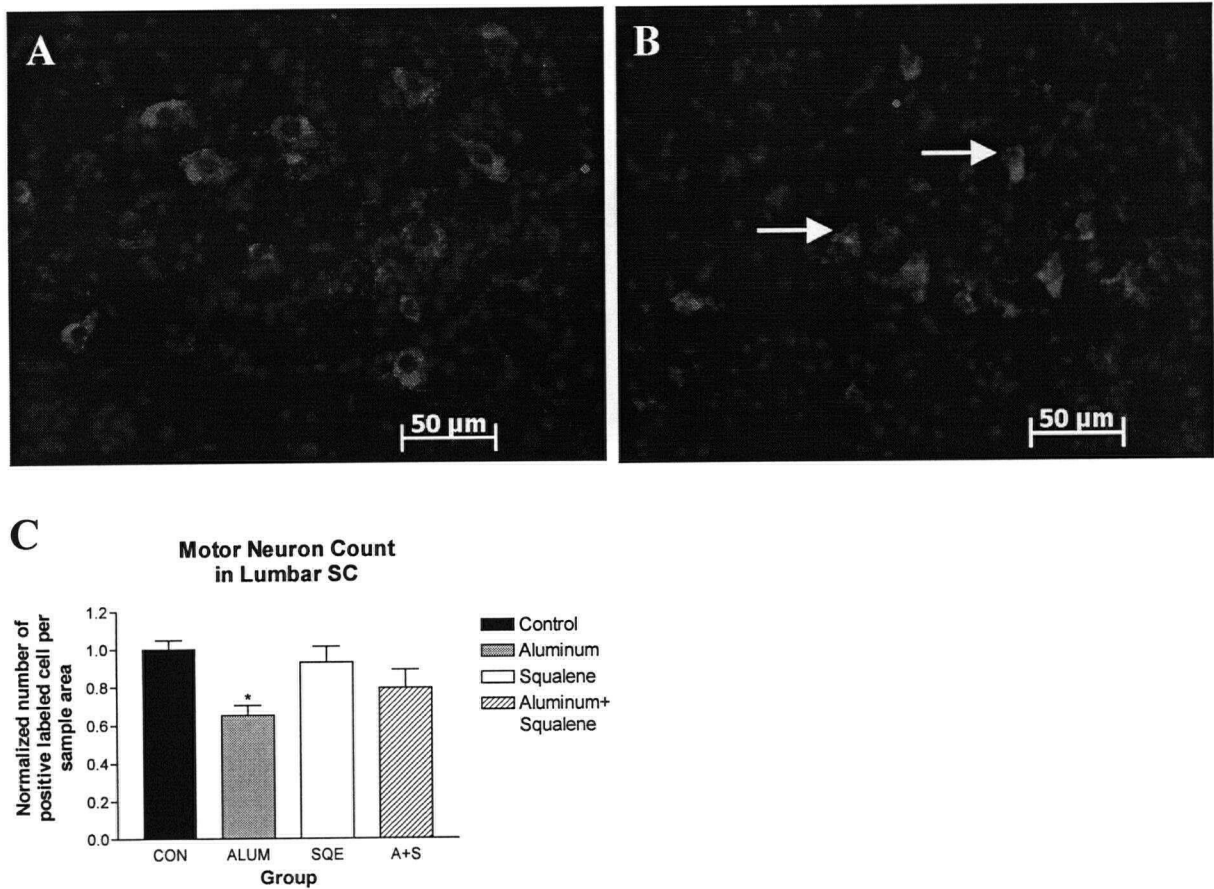


Figure 9. Choline acetyltransferase (ChAT) fluorescent labeling in ventral horn of lumbar spinal cord. A: Control section shows ChAT labeling of motor neurons (20x magnification). B: Aluminum injected animal shows decreased ChAT labeling and abnormal morphology of motor neurons (white arrows) compared to controls (20x magnification). Scale bar = 50 μ m. C: Only cells positively labeled with ChAT were counted as motor neurons (n=32, 8 per group). Mice injected with aluminum hydroxide showed a statistically significant decrease in motor neuron number (35%) compared to controls. There was no significant difference in motor neuron counts between all other groups compared to controls. Data are means \pm S.E.M * p <0.001 versus control mice using one-way ANOVA.

Figure 10

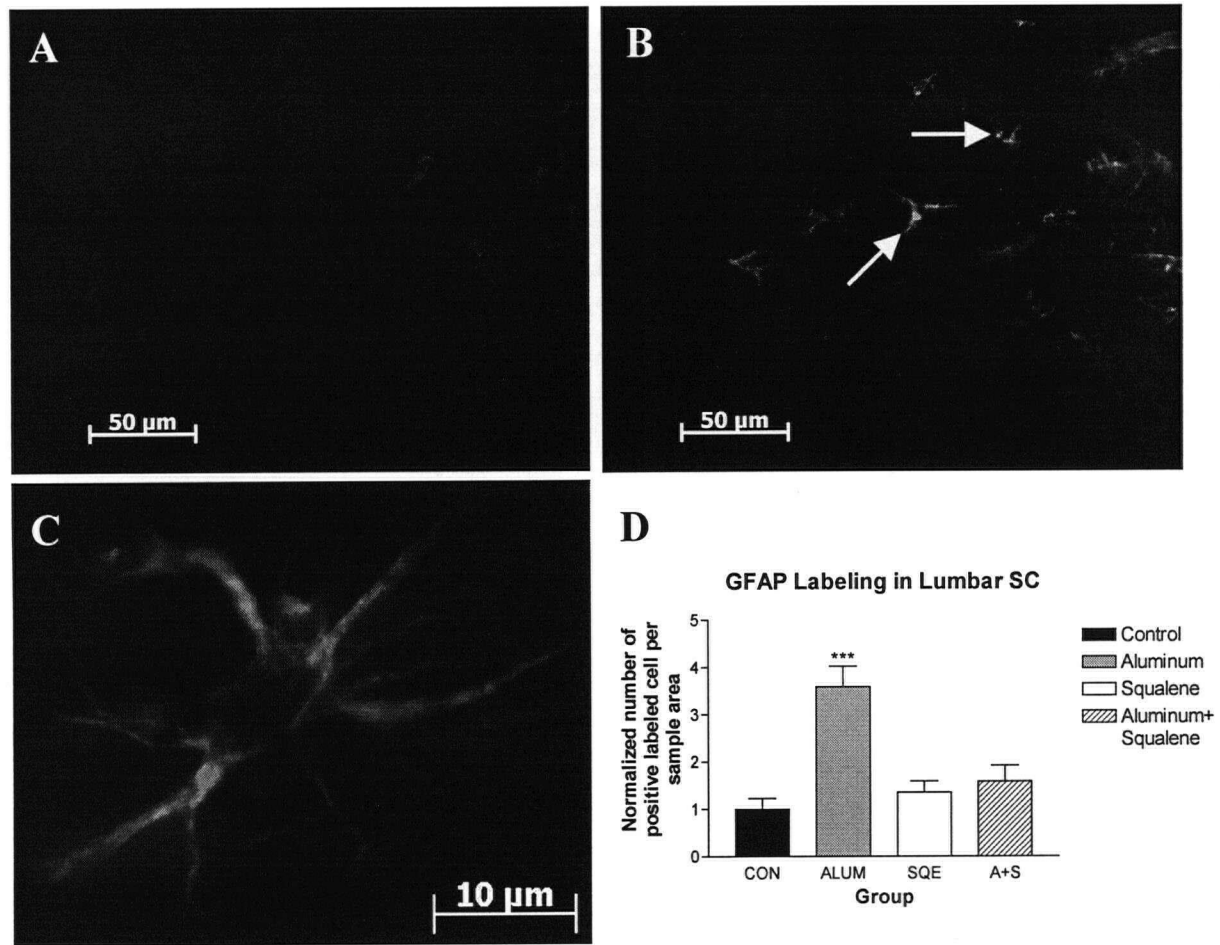


Figure 10. GFAP fluorescent labeling in ventral horn of lumbar spinal cord. A: Control sections show little GFAP labeling. B: Sections from mice injected with aluminum hydroxide show increased GFAP labeling and greater number of astrocytes (white arrows) compared to controls (A-B, 40x magnification). Scale bar = 50 μ m. C: Astrocyte from aluminum injected mouse observed under 100x magnification. Scale bar = 10 μ m. D: Normalized cell counts for GFAP labeling of astrocytes in ventral horn of lumbar spinal cord (n=32, 8 per group). Squalene treated animals show a small increase in GFAP labeled astrocytes when compared to controls. Animals treated with both aluminum hydroxide and squalene showed a larger increase in astrocyte cell number while mice injected with aluminum showed the greatest increase in GFAP labeled astrocytes (350%). Data are means \pm S.E.M. *** $p < 0.001$ versus control mice using one-way ANOVA.

Figure 11

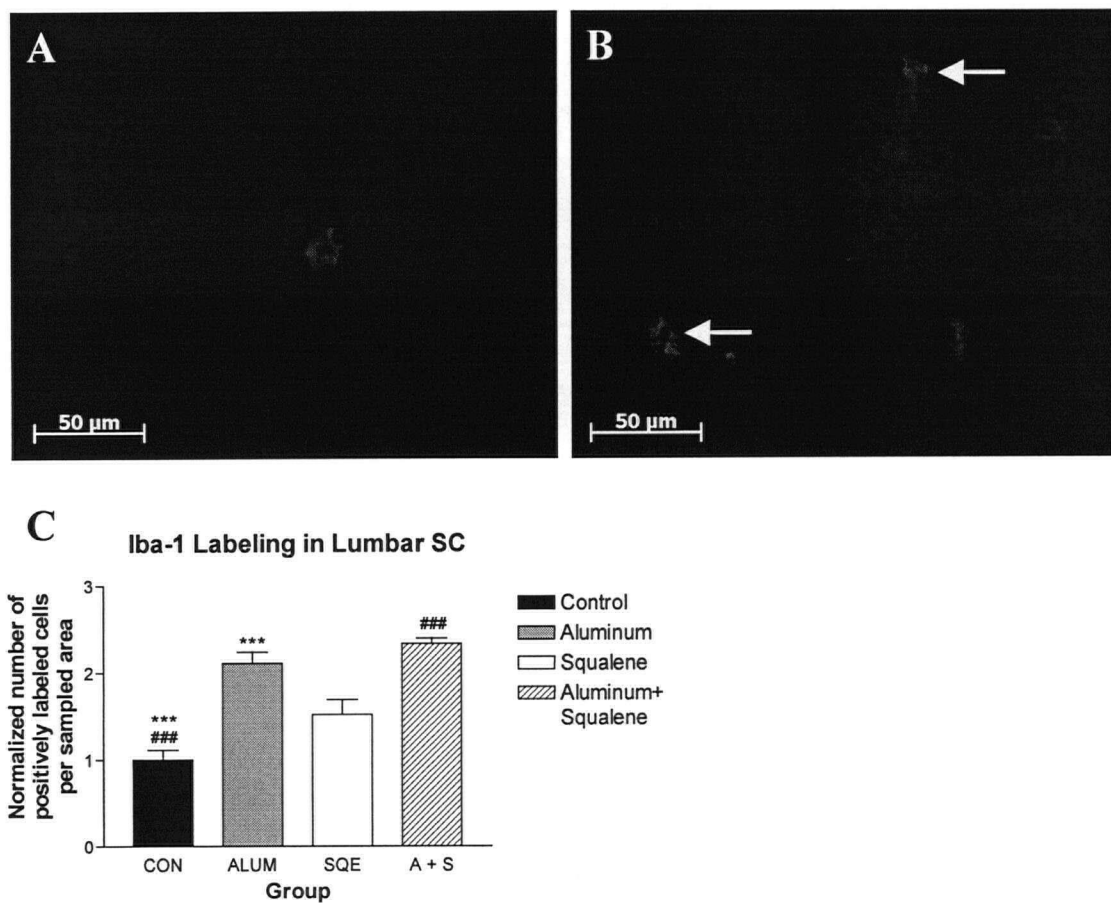


Figure 11. Iba-1 immunoreactivity in ventral horn of lumbar spinal cord. A: Preliminary staining with Iba -1 shows little immunoreactivity in control mice. B: Sections from mice injected with aluminum hydroxide show increased Iba-1 labeling and greater number of microglia (white arrows) compared to controls (A-B, 40x magnification). Scale bar = 50 μ m. C: Normalized cell counts for Iba-1 labeling of microglia in ventral horn of lumbar spinal cord (n=16, 4 per group). Squalene treated animals show a small increase in Iba-1 labeled microglia when compared to controls. Significant increases in microglial number were observed in animals treated with aluminum hydroxide (211%) or both aluminum hydroxide and squalene (233%) compared to controls. Data are means \pm S.E.M. ***, ### $p < 0.001$ versus control mice using one-way ANOVA.

Figure 12

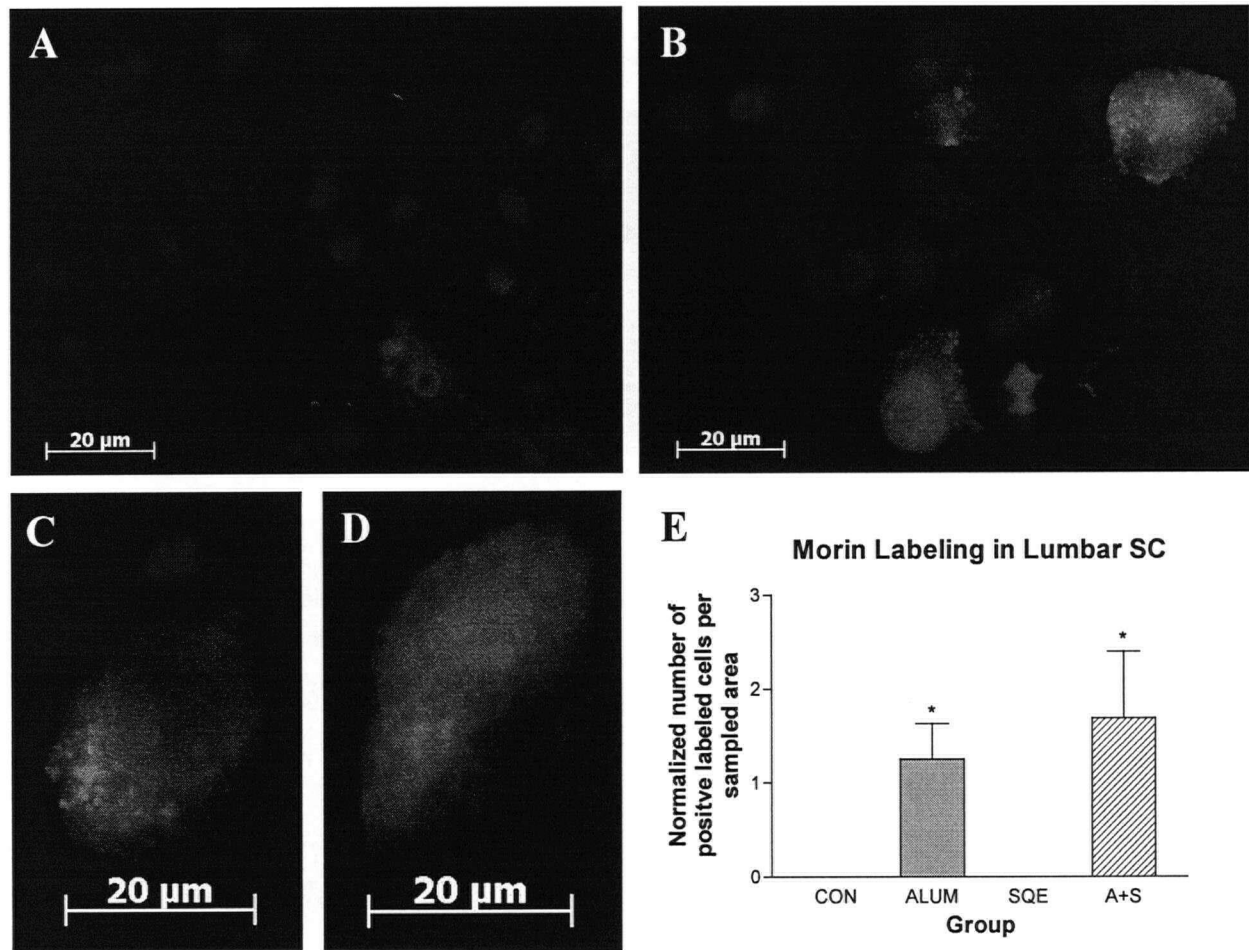


Figure 12. Morin fluorescent labeling in ventral horn of lumbar spinal cord. A: Control sections show no Morin fluorescent labeling. Scale bar = 20 µm. B: Animals injected with aluminum show significant Morin labeling compared to squalene and control groups. Scale bar = 20 µm. C, D: Cells positively labeled with Morin from animals injected with both aluminum and squalene. Scale bar = 20 µm. E: Cell counts for Morin positive cells in ventral horn of lumbar spinal cord (n=16, 4 per group). Both animal groups treated with aluminum show positive Morin fluorescence indicating the presence of aluminum in some cells. Data are mean ± S.E.M. One-way ANOVA analysis revealed a significance level of * $p < 0.05$; post Tukey test did not reveal any significance between groups.

Figure 13

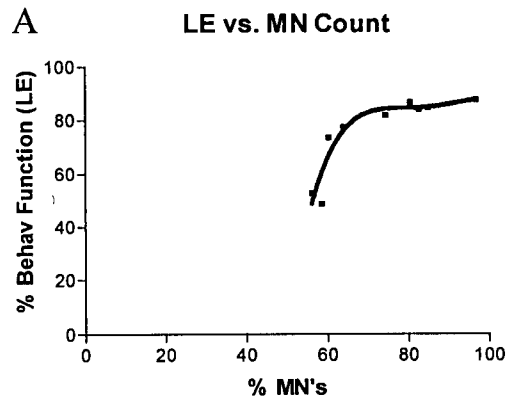


Figure 13. Relationship of leg extension score versus motor neuron count. A: Graph shows the inverse relationship of leg extension scores with percentage of motor neuron number present. This data was collected from several previous experiments conducted by the Shaw lab at UBC (Wilson, unpublished) using an environmental mouse model of motor neuron disease (ALS-PDC). Graphs depicts that mice can have up to approximately 40% motor neuron loss and still not show any significantly reduced leg extension scores.

Table 4

Comparison of human ALS and GWI symptomology with outcomes observed in aluminum injected mice.			
Symptoms	ALS*	GWI†	Aluminum injected mice
Muscular strength and endurance loss	+	+	+
Enhanced anxiety	+	+	+
Memory impairment	+	+	+
Dermatitis		+	+

*Bromberg, 2002. †Haley et al., 1997.

Table 4. Summary of human ALS and GWI symptoms compared with outcomes observed in aluminum injected mice. This table also outlines the similarities between human ALS and Gulf War illness.

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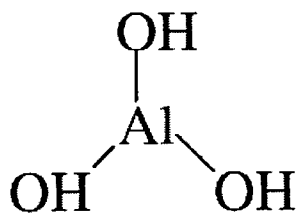
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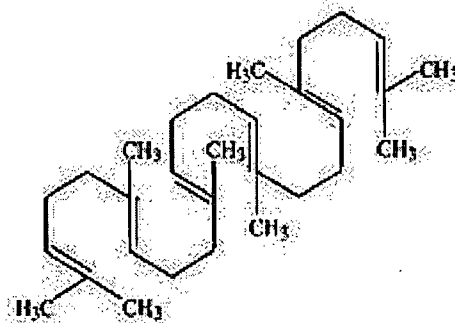
Appendix 1

A



ALUMINUM HYDROXIDE

B



Appendix 1. Aluminum hydroxide and squalene. Chemical structures of aluminum hydroxide (A) and squalene (B).