ALUMINUM ADJUVANT LINKED TO GULF WAR SYNDROME INDUCES MOTOR NEURON DEATH IN MICE

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ABSTRACT

Gulf War Syndrome (GWS) affects a high percentage of veterans of the 1991 conflict, but its origins remain unknown. One neurological complication of GWS is an increased incidence of amyotrophic lateral sclerosis (ALS). While many environmental factors have been linked to GWS, the role of the anthrax vaccine administered to deployed troops has come under increasing scrutiny. Among the vaccine's potentially toxic components are the adjuvant aluminum hydroxide and squalene. To examine whether these materials might contribute to neurologic toxicity, we injected young male colony CD-1 mice with these adjuvants at doses equivalent to those given to service personnel. Mice were subjected to a battery of motor and cognitive behavioral tests over a six month period. Following sacrifice, CNS tissue was examined using immunohistochemistry for evidence of neural death. Behavioral testing showed both motor and cognitive functions were impacted by the tested adjuvants to differing degrees. Apoptotic neurons were identified in lumbar spinal cord and motor cortex in the groups receiving the adjuvants. Aluminum injected animals also showed a significant increase of astrocytes in the lumbar spinal cord. Our findings suggest a possible role for either or both compounds in some neurological features associated with GWS.

KEY WORDS: ALS, GULF WAR SYNDROME, ADJUVANT, ALUMINUM HYDROXIDE, SQUALENE, ANTHRAX, VACCINE, NEUROTOXICITY.

INTRODUCTION

Gulf War Syndrome (GWS) is a cluster of illnesses in veterans of the Persian Gulf War (1990–91) characterized by a group of variable and nonspecific symptoms such as fatigue, emotional disorders, muscle and joint pains, headaches, memory loss, and posttraumatic stress reactions (Haley et al., 1997; Fukuda et al., 1998). Previous studies conducted on Gulf War veterans by the U.S. Department of Defense, the U.S. Department of Veteran Affairs and the U.K. Gulf War Research Illness Unit have established a strong link between Gulf War service and the occurrence of Gulf War Syndrome (GWS) (Hom et al., 1997; Unwin et al., 1999; Kang et al., 2002; Wolfe et al., 2002; Dyer, 2004).

Recent studies have established a correlation between Gulf War service and a neurological cluster of amyotrophic lateral sclerosis – Gulf War Syndrome (ALS-GWS) (Charatan, 2002; Horner et al., 2003; Weisskopf et al., 2005). According to a nationwide study by the U.S. Department of Veterans Affairs, deployed veterans of the Persian Gulf War are more than *twice* as likely to develop ALS than non-deployed veterans and the civilian population (Samson, 2002). The most unique feature of this new cluster is that the victims are younger than usual ALS patients (Haley, 2003). Due to the overlapping symptomatology seen in GWS and ALS, GWS can be partially classified as a neurological illness that may carry an ALS component. One major cluster of ALS in the Western Pacific, amyotrophic lateral sclerosis-parkinsonism dementia complex (ALS-PDC) (Kurland, 1988; Murakami, 1999), suggests an environmental cause. Gulf War

Syndrome ALS may comprise another cluster of the disease and as such may provide clues to the etiology of ALS.

Epidemiological studies have suggested several potential environmental factors in GWS such as exposure to depleted uranium, nerve gas, organophosphates, vaccinations, heavy metals, and bacteria infections (Abou-Donia et al., 1996; Taylor et al., 1997; Kurt, 1998; Hodgson and Kipen, 1999; Sartin, 2000; Hotopf, 2000; Ferguson and Cassaday, 2001-2002; Nicolson et al., 2002). Of these, vaccines are highly suspected because multiple epidemiological studies have found positive correlations between vaccinations and GWS, and because some nondeployed but vaccinated troops have developed illnesses identical to GWS**.** In particular, the anthrax vaccine is a major suspect in GWS as it contains two materials of particular interest from a neurological perspective: aluminum hydroxide and the lipid polymer squalene (which has been found in the vaccine at low concentrations) (Sasaki et al., 1992); (Sahin et al., 1994; Schumm et al., 2002) forms of both having been implicated in neurological disease (Garruto et al., 1989; Wagner-Recio et al., 1991). Aluminum and squalene have been linked to neurotoxicity in a number of other studies (Bilkei-Gorzo, 1993; Gajkowska et al., 1999; Nass, 2002), and antibodies to squalene have shown up in those with GWS (Asa et al., 2000). LD_{50} values (via subcutaneous injection) for either compound have not been published to date (see J.T. Baker Material Safety Data Sheets).

METHODS

Experimental animals, diet and tissue collection

Adult CD-1 male mice were used in the study (3 months old at experiment onset, weighing approximately 35g). Younger animals were deliberately chosen to mimic the age of onset in young Gulf War veterans (Haley, 2003). The control group contained 10 animals and each adjuvant group contained either 10 or 11 animals. All animals were housed solitarily at the Jack Bell Animal Care Facility, where an ambient temperature of 22^{o}C and a 12/12hr light cycle were maintained. All mice were consistently fed Purina® mouse chow ad libitum. Mice were subjected to several behavioral tests including wire mesh hang (2x/week), open field (1x/week), and water maze (1x/week) over a period of six months. The behavioral tests were conducted in the same room over a total period of 24 weeks. The order of animals tested was randomized for each trial. At sacrifice, the CNS tissue was collected for histological examinations. Brains and spinal cords of mice were obtained from both adjuvant and control groups upon sacrifice by perfusion with 4% paraformadehyde (PFA). Fixed brains and spinal cords from adjuvant and control mice were transferred to a 30% sucrose/PBS solution overnight and kept frozen until sectioning. The CNS sections were cryoprotected in 30% ethylene glycol-20% glycerol-dibasic and monobasic sodium phosphate solution and kept frozen at –20^{o}C. Brains were sectioned into 30 µm slices and spinal cords were sectioned at 25 µm in the transverse plane on a cryostat mounted in Tissue-Tek O.C.T compound (Sakura, Zoeterwoude, Netherlands).

Adjuvants

Alhydrogel®, an aluminum hydroxide ($Al(OH)_3$) gel suspension is manufactured by Superfos Biosector a/s (Denmark) and supplied by SIGMA, Canada. MPL® + TDM + CWS (Monophosphoryl Lipid A, syntheitic Trehalose Dicorynomycolate, and cell wall skeleton of mycobacteria), a commercial squalene ($C_{30}H_{50}$) containing adjuvant was manufactured by Corixa Corporation (Seattle, USA). Both were supplied by SIGMA, Canada.

To calculate equivalent to human dosages of aluminum hydroxide and squalene for our experiments, we used the following information: The AVA vaccine for human use is made by Bioport Corporation, of Lansing, Michigan. According to product data sheets from the Michigan Biological Products Institute anthrax vaccine insert (Bioport's predecessor) a single dose of vaccine contains 2.4 mg of aluminum hydroxide (equivalent to 0.83mg of aluminum). Based on an assumed average human body weight of 80 kg, the amount per kg body weight is approximately 30µg/kg. Soldiers or civilians receiving the vaccine would have received between 30µg/kg (1 injection) up to 180ug/kg if 6 injections were received. Bioport Corporation denies the addition of squalene in the formulation. The company producing the vaccine during the 1990/1991 periods, Michigan Biological Products Institute, also denied use of squalene in the formulation of the product. However, antibodies to squalene has been found in blood samples from patients with GWS and in higher in titers than those produced naturally by the body ((Asa et al., 2000)), suggesting that it was present in at least some anthrax vaccine lots in use at

the time. Current vaccines in use outside the United States employ a squalene containing adjuvant oil emulsion. MF59, an adjuvant in experimental influenza vaccines (Chiron Corporation) use a 5% squalene concentration. 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 70kg human, the amount per injection would be approximately 21.5µg (0.3µg/kg) for one injection, as much as 86µg (1.2µg/kg) for a full series of 4 injections.

The adjuvant injections in our 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 approximate average rather than the range of 1 to 4 injections in the human subjects. Based on the human values above, mice receiving aluminum hydroxide received two doses of 50µg/kg (suspension) in a total volume of 200µL sterile PBS (0.9%). Mice receiving squalene got the equivalent dose of 2% squalene suspension (MPL® + TDM + CWS) in PBS. Mice in the aluminum hydroxide + squalene group had both adjuvants administered the same PBS volume. Controls were injected with 200µL PBS.

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.

Immunization

Animals received two injections (two weeks apart) of aluminum hydroxide, squalene, aluminum hydroxide and squalene or PBS. The adjuvants were then administered by subcutaneous injection at the back of the neck. This immunization protocol mimicked the anthrax vaccine dose schedule set by the Anthrax Vaccine Immunization Program (AVIP) except for the location of the injection.

Behavioral Testing

Wire Mesh Hang

The wire mesh hang was used to test for muscular strength and endurance (Crawley, 2000). The wire mesh hang consisted of a 6-inch wire mesh that was suspended 40 cm high. Mice placed onto the wire grid and inverted for a maximum period of 60 s. Latency to fall was measured and recorded three times per week.

Open Field

The 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 2 inches thick. An overhead video camera was used to record mouse locomotion in the open field environment. The investigator counted the number of squares crossed in a measured area (outside, inside and center perimeters) over 5 min. Anxiety, or fear-related behavior, is seen when the mouse remains in the corners or near

the edges of the arena (thigmotaxis) rather than moving out in the center of the arena (Crawley et al., 1997). Testing was conducted once a week for the duration of the experiment.

Morris Water Maze

The water maze was used to evaluate spatial and reference 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 the water level. The mice were trained for 4 d at 3 trials/d prior to the injection paradigm. 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 60 s, 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, errors were counted if the mouse fully entered the incorrect arm.

Immunohistochemistry

Neuronal Nuclei (NeuN) and activated caspase-3 labeling

Mouse NeuN antibody (Chemicon International; Temecula, CA, 1:300) was used to identify neurons containing NeuN, a DNA-binding and neuron-specific nuclear protein (Mullen et al., 1992; Wolf et al., 1996). Free-floating sections were rinsed in 10% tris-EDTA buffer and microwaved for 10 min. After heating, sections were allowed to cool for 20 min. Sections were then incubated in working solution of MOM Mouse IG

Blocking Reagent (MOM kit, Vector Laboratories) for 1 h. Sections were immersed in MOM Diluent solution for 5 min and incubated in primary NeuN antibody for 30 min at room temperature. Sections were then incubated in MOM Biotinylated Anti-Mouse IgG Reagent for 10 min and incubated with Fluorescein Avidin DCS for 5 min, then blocked with 10% NGS (normal goat serum) for 1 h. Sections were incubated with rabbit anti-activated caspase-3 antibody (Promega; Madison, WI, 1:250) overnight and AlexaFluor 546™ for 30 min at room temperature (Molecular Probes; Eugene, OR, 1:500) to detect cells undergoing apoptosis (Duan et al., 2003). Sections were mounted with fluorescent DAPI (4',6 diamidino-2-phenylindole, Vector Laboratories). 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.

Choline acetyltransferase (ChAT) labeling

ChAT antibody (AB144P, Chemicon International; Temecula, CA, 1:100) was used to identify cholinergic neurons in brain and spinal cord and serves as a specific marker for motor neurons (Wetts and Vaughn, 1996; Maatkamp et al., 2004). Fluorescent immunolabeling was performed on free-floating sections and pretreated in 0.5% Triton X-100 in buffer for 2 x 15 min. Sections were then blocked in 5% NGS (normal goat serum) with 5% BSA (bovine serum albumin) for 3 hours, 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 h each in rabbit anti-goat IgG antibody (1:200; DuoLuX™,

Elite ABC Kit, Vector Laboratories) at room temperature and mounted with fluorescent DAPI (4',6 diamidino-2-phenylindole, Vector Laboratories).

Glial fibrillary acidic protein (GFAP) labeling

GFAP is a member of the class III intermediate filament protein family. It is heavily, and specifically, expressed in astrocytes and certain other astroglia in the central nervous system (Lee et al., 1984; Tohyama et al., 1991; Lee et al., 1984). Anti-Glial Fibrillary Acidic Protein Rat monoclonal antibody (345860, Calbiochem, San Diego, CA, 1:100) was used to identify astrocytes in lumbar segment of animal spinal cord. Fluorescent immunolabeling was performed on free-floating sections and pretreated in 0.5% Triton X-100 in buffer (PBST) for 2 x 5 min. Sections were then blocked in 10% NGS + 1%BSA in PBST for 2 hours, then incubated with primary antibody rat-anti-GFAP (in PBST with 1%NGS + 1%BSA) at 10ug/ml (1:100) in a humidified chamber for overnight at room temperature (23°C). Sections were then incubated for 1 hour in anti-rat Fluorescein Isothiocyanate (FITC) antibody (1:200 dilution in PBS, Serotec Laboratories) incubate for at room temperature and mounted with fluorescent DAPI (4',6 diamidino-2-phenylindole, Vector Laboratories).

Microscopy

Brain and spinal cord sections processed with fluorescent materials were viewed with a Zeiss Axiovert microscope at 40x magnification and 100x magnification under oil. When these fluorescent markers are excited they can be easily detected by fluorescent microscopy. DAPI (blue) was viewed with a 359/461 nm absorption/emission filter,

Alexa Fluor 546™ (red), and rabbit IgG DuoLuX™ (red) was viewed with 556,557/572,573 nm filter and FITC was viewed with a 490,494/520,525 nm filter. Images were captured using AxioVision 4.3 software. Brains and spinal cords used for histology were chosen randomly from each group. Cerebral cortices and lumbar cord slices were sampled per animal per histological experiment.

NeuN and active caspase-3 quantification

NeuN and active caspase-3 assays were performed to examine CNS tissue for evidence of neurodegeneration. NeuN staining was used to label neuronal cells and activated caspase-3 to measure apoptosis. Multiple brain and lumbar spinal cord sections (n=3,8) from each mouse were captured as previously described. Fluorescent intensity levels of NeuN and activated caspase-3 were used to identify specific antibody labeling. Double labeling of NeuN and activated caspase-3 indicates neurons undergoing programmed cell death. Stained sections included primary motor cortex, red nucleus, substantia nigra, dentate gyrus layer of hippocampus and lumbar spinal cord. Regions of interest (ROI) were defined using landmarks from stereotaxic mouse brain and spinal cord atlases (Sidman et al., 1971; Paxinos and Franklin, 2001). All sections were randomized and counted in an unbiased manor. Cell counts included total number of cells labeled either NeuN, activated caspase-3, or both markers (double labeling) and were counted under a 40x objective lens, then compared for quantification. Five mice from each group were used for lumbar spinal cord histology and five from each group for brain histology.

Choline acetyltransferase (ChAT) quantification

ChAT staining was used to identify motor neurons by labeling choline acetyltransferase; an enzyme used by motor neurons to synthesize the neurotransmitter acetylcholine (ACh). Lumbar spinal cord sections (n=8) from each mouse were captured and ROIs defined using the methods previously described. Ventral root motor neurons were counted under a 40x objective lens and the experimenter was blind to the counts. All motor neurons in the field of view were counted in the results for quantification. Eight mice from each group were used.

Glial fibrillary acidic protein (GFAP) quantification

GFAP stains reactive rodent and human brain astrocytes induced by a variety of central nervous system injuries. Lumbar spinal cord sections (n=8) from each mouse were captured and ROIs defined using the methods previously described. Only positively GFAP labeled astrocytes in the ventral horn of the grey matter where included in the counting. Counts were conducted under a 40x objective lens and the experimenter was blind to the counts. All astrocytic cells in the field of view were counted in the results for quantification. Eight mice from each group were used.

Anti-squalene antibody assay (ASA)

Squalene was diluted 10-, 100-, 1000-, and 10,000-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 min prior to the addition of 5 µl of mouse sera (1:100 to 400 dilution) followed by a further 90-min 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 min 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 a 60-min 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-min incubation, the strips were washed, as above, and 2 ml of buffered saline containing 30% methanol and the substrate 0.6 mg/ml 4-chloro-1-napthol, 0.03% hydrogen peroxide; pH 7.4) was added. The reaction was allowed to proceed for 15 min and was stopped by rinsing the strips in distilled water. The strips were allowed to air-dry for visual scoring on a scale of 0 to 4.

Statistics

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

RESULTS

We used a combination of motor and cognitive behavioral tests over a six-month period, including tests for muscular strength and coordination (wire mesh hang), spontaneous locomotor activity and anxiety (open field) and reference memory (radial arm water maze).

The greatest overall effects were seen in mice injected with aluminum hydroxide. These mice showed a significant decrease in muscular strength and endurance (-50%) compared to controls (Fig. 1A). Squalene (in the form of an adjuvant system, MPL® + TDM + CWS; see Adjuvants in Methods) 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. Aluminum injected mice showed a significant increase in anxiety levels (+38%), measured by open field testing, compared to controls (Fig. 1B). The squalene group also showed a small increase in anxiety after week 20 but these results did not achieve significance. There was no difference in anxiety levels between the combined group and controls.

Assessment of cognitive performance on a Morris water maze showed that mice injected with both adjuvants had significant late stage memory deficits with an increase in the number of errors after week 20 (4.3 errors) compared to controls (0.2 errors) (Fig. 1C).

Mice injected with squalene (0.9 errors) or aluminum hydroxide (1.2 errors) also showed an increase in the number of errors after week 20.

In addition to behavioral changes, physiological changes were observed. Four of the ten mice injected with both adjuvants developed an allergic skin reaction (i.e. dermatitis) approximately 0.5 cm diameter region around the injection site. Hair loss at the injection site (0.5 cm to 1.0cm diameter region around the injections site) was also visible in all groups; 3/10 mice from the combined group, 2/10 from the aluminum hydroxide group, 4/10 from the squalene group, and no control animals (injected with PBS) developed hair loss in this area.

Previous studies of ALS pathology have shown increased numbers of dying cells undergoing apoptosis, a normal form of programmed cell death (Troost et al., 1995; Martin, 1999). To measure apoptosis, we used colabeling of NeuN, a neuronal marker; and activated caspase-3, a key mediator of the apoptotic cell death pathway (Mullen et al., 1992; Wolf et al., 1996; Duan et al., 2003). Both NeuN and activated caspase-3 primary antibodies were tagged with fluorescent secondary antibodies (Fluorescein Avidin DCS and AlexaFluor 546™) in order to provide fluorescent labeling.

Mice injected with PBS showed little or no activated caspase-3 labeling in ventral lumbar spinal cord (Fig 2C, E, G; 3A). In contrast, mice injected with aluminum hydroxide showed a significant 155% increase in activated caspase-3 labeling alone and a significant 133% increase in double labeling (Fig. 2D, F, H; 3A). Activated caspase-3

was also increased in the squalene group as well as the combined aluminum and squalene group, but quantified cell counts were not significant. The difference between activated caspase-3 and NeuN labeling between groups suggests that other cell types are undergoing apoptosis. Aluminum injected mice also showed a significant reduction in motor neurons (-35%) compared to controls (Fig. 3B). The squalene and combined group also showed a reduction in motor neuron number that did not achieve significance (Fig. 3B).

In addition to the spinal cord, we also examined brain structures involved in motor function and movement. NeuN and activated caspase-3 immunohistology was performed on the primary motor cortex and brainstem (substantia nigra and red nucleus for evidence of apoptotic neuropathy), since these areas are affected in ALS (Sasaki et al., 1992; Eisen and Weber, 2001; Tsuchiya et al., 2002). Quantitative analysis of NeuN labeling showed insignificant differences between groups indicating comparable numbers of labeled neurons in all treatment groups (Fig 3A, C-F). Mice injected with aluminum hydroxide showed a significant increase in activated caspase-3 (+92%) and double labeling (+85%) in primary motor cortex compared to controls (Fig. 3C). The squalene and combined group showed a small increase in both labels but it was not significant. Cell counts performed in the red nucleus show increased activated caspase-3 and double labeling in both aluminum groups, but this did not achieve statistical significance (Fig. 3D). Analysis of the substantia nigra region did not reveal any differences in labeling between all groups (Fig 3E). In the hippocampus, cell counts conducted on the polymorph layer of

the dentate gyrus (DG) showed an increase in double labeling for squalene and combined groups but it was not statistically significant (Fig. 3F).

Previous studies have shown the increased presence of astrocytes, cells that express inflammatory markers and downregulate glutamate transporters, in human cases and animal models of ALS (Nagy et al., 1994; O'Reilly et al., 1995; Levine et al., 1999; Barbeito et al., 2004). To examine the number of astrocytes present in the spinal cord we used a GFAP primary antibody to label astrocytic cells throughout the lumbar segment (Fig.4A, B). Animals treated with squalene, showed a small increase in the number of astrocytes present when compared to controls but this was not significant (Fig. 5). Mice injected with both adjuvants also showed a larger increase in positively labeled astrocytes, but this was also not statistically significant. The aluminum injected group showed the largest presence of astrocytes (approximately 3.5 times greater than controls) achieving statistical significance (Fig 5).

To determine whether or not mice injected with adjuvants present in anthrax vaccines develop antibodies to squalene, sera from animals injected with a squalene containing adjuvant, with aluminum hydroxide or with both adjuvants were tested blindly in an ASA assay adapted for mice. Two of ten control animals possessed ASA in the first serum specimen taken at 4 weeks (2 weeks post second injection). A higher number of animals, 4/10, injected with squalene possessed detectable levels of ASA at this time point, however this difference was not statistically significant. 3/11 animals injected with

aluminum hydroxide and 1/10 injected with both adjuvants possessed ASA. The presence of ASA was generally stable over time in individual animals tested. However, one animal that had been injected with both adjuvants developed ASA at a later time point (24 weeks post last injection).

DISCUSSION

Using the anthrax vaccine adjuvant aluminum hydroxide and squalene (not a licensed component in North America) under minimal conditions (1-4 injection range), we investigated the behavioral and neuropathological effects of these adjuvants in mice. Our data suggest that the aluminum hydroxide adjuvant induces both behavioral and motor deficits and the loss of motor neurons and increased presence of astrocytes in spinal cord and neuronal apoptosis in the primary motor cortex while also affecting the red nucleus region of the brain. The squalene adjuvant alone produced a small change in locomotion and anxiety testing, but the histological results were not significant in the current experiments. The combination of both adjuvants showed a significant memory deficit with some indications of neuronal apoptosis in the red nucleus and DG region of the hippocampus.

Several possibilities may explain the neurotoxic effects of these adjuvants demonstrated in the current results. First, the adjuvant compounds may exert direct toxicity on some neural cell populations in the CNS (Bilkei-Gorzo, 1993; Gajkowska et al., 1999). Aluminum in particular has long been associated with neuronal degeneration and

neurodegenerative diseases (Rao et al., 1998; Savory and Garruto, 1998), and aluminum adjuvanted vaccines have been shown to increase aluminum levels in the murine brain (Redhead et al., 1992; Sahin et al., 1994). Aluminum treated animals have also shown severe anterograde degeneration of cholinergic terminals in cortex and hippocampus (Platt et al., 2001). Squalene has been shown to cause swelling in astrocytic processes (Gajkowska et al., 1999).

Alternatively, the adjuvants may induce an indirect type of toxicity by stimulating an 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 the Th2 predominance, versus Th1 (T helper cell type 1), and maximize Th2 immunogenicity (Rook and Zumla, 1997; Rook and Zumla, 1998). Both aluminum hydroxide and squalene have previously been shown stimulate a Th2-cytokine response (Valensi et al., 1994; Brewer et al., 1999). A latest study using inbred and outbred mice injected with recombinant protective antigen (rPA) vaccine and challenged with Bacillus anthracis, found that both mouse strains displayed a predominantly Th2 biased immune response (Flick-Smith et al., 2005). Such a Th1 to Th2 shift could stimulate autoimmune processes. A recent study of blood samples from Gulf War veterans, however, showed evidence for Th1 immune activation (Skowera et al., 2004). Alternatively, the observed effects of aluminum hydroxide and squalene (or other components in the squalene

containing adjuvant) in our study could result from direct action on neuronal cells in the CNS.

In the present study, the combination of aluminum hydroxide and squalene seemed to have less effect on motor behavior and anxiety testing than either aluminum hydroxide or squalene alone. The possibility of competing effects on immune response cannot be discounted and deserve further investigation. It is notable that while both compounds are present in the anthrax vaccines administered to deployed service personnel, the company making these vaccines has not confirmed the addition of squalene. Although squalene has adjuvant properties, we are not claiming it was deliberately added to adjuvant the vaccine, and it is not a licensed ingredient of the vaccine. However, blood samples from patients with GWS have been reported to contain anti-squalene antibodies in much higher in titers than those produced naturally (Asa et al., 2000), suggesting that it was present in at least some anthrax vaccine lots in use at the time. Conversely, although anti-squalene antibodies have been found in Gulf War vets, squalene is a component of human bodies and such antibodies may be unrelated to the deliberate injection or ingestion of squalene (which can be found in cosmetics and various foods).

Previous studies suggested that anti-squalene antibodies are present in the serum of veterans with GWS at a greater frequency than the general population (Asa et al., 2000). The presence of low levels of squalene in certain lots of anthrax vaccine correlated significantly with the presence of ASA (Asa et al., 2002). Our current studies using CD-1

mice were unable to provide additional evidence for the role of ASA in the neurological defects of GWS. Although ASA were detected in a subset of mice infected with vaccine adjuvants, the study was complicated by the presence of ASA in mice that had not been injected with squalene. 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). However, of the mouse strains tested BALB/c, B10.Br and C57BL/6, fewer than 12% of animals possessed antibodies naturally by 10 months of age, which is older than any of the CD-1 mice used in the current study. Forty percent of CD-1 mice injected with squalene containing adjuvant possessed ASA. It is possible that this stain of mice develops antibodies to squalene as a natural consequence of aging at an accelerated rate, compared to the other mouse strains previously tested by Matyas and coworkers. Differences in the sensitivities and specificities of the ASA assays could also contribute to differences in these observations.

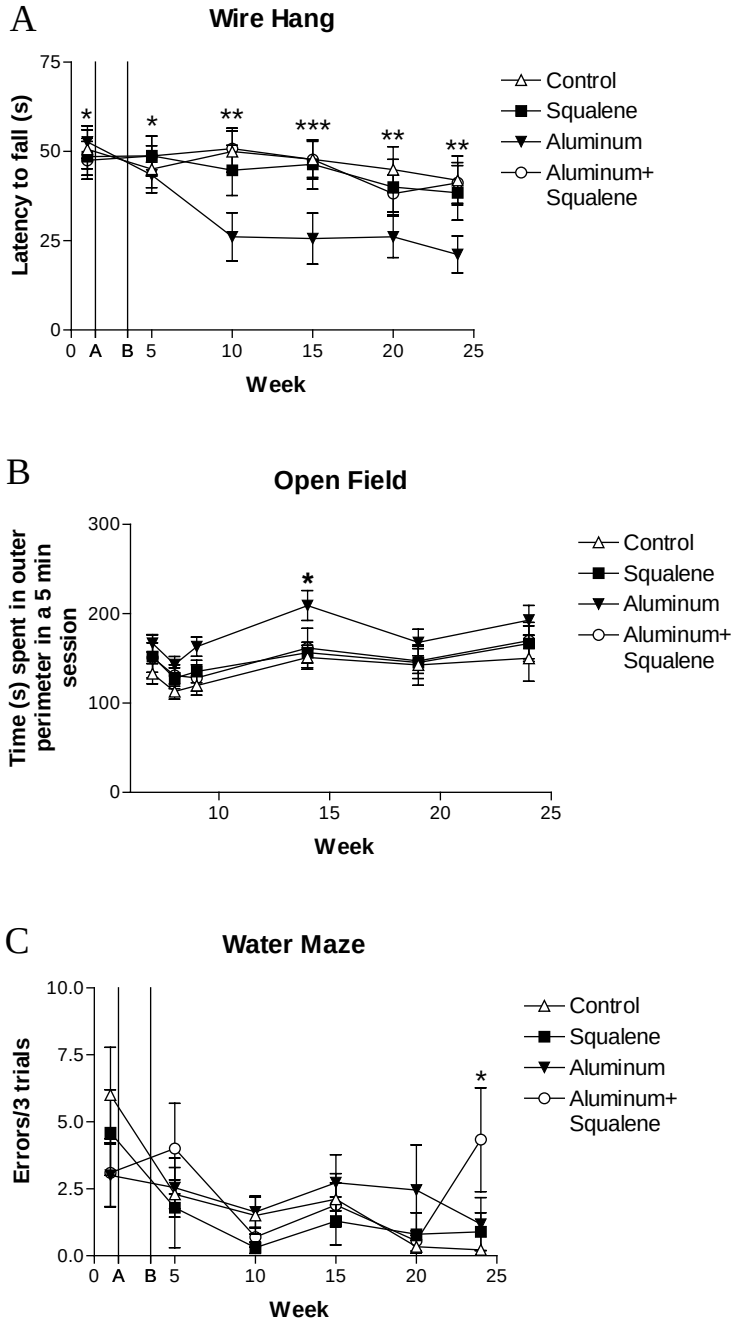
Our findings demonstrate that two anthrax vaccine components appear to have negative impacts on motor behavior and motor neuron survival and give outcomes that conceivably may resemble GWS-ALS. It is important to note that in this study we studied only these two components (aluminum hydroxide and squalene containing adjuvant) and not the vaccine itself. Although several animal studies using the anthrax vaccine have been published (Ivins et al., 1995; Fellows et al., 2001; Williamson et al., 2005), neither of these experiments examined neurological outcomes or behavioral side effects.

In recent human studies, there is strong suggestive evidence of a vaccine link to Gulf War syndrome. A study performed on vaccine-free French soldiers who served in the Gulf War show that they are relatively free of illness compared to their U.S. and U.K. allies who received multiple vaccinations (Brown, 2002). The disturbing outcomes from animal and human vaccine studies have fueled a strong urgency from members of the scientific community to revoke current anthrax vaccine licensing. A recent submission to the U.S. Department of Health and Human Services states that the efficacy and safety of the anthrax vaccine in humans have not been scientifically demonstrated, and recommends that licensing not proceed (Nass, 2002; Nass et al., 2005).

The current results show that further studies on the potential neurotoxic properties of these compounds are needed, especially given that the current anthrax vaccine is more concentrated now and the immunization schedule carries a higher number of injections than used in 1990-1991. In addition, the continued use of such adjuvants in various vaccines (i.e., Hepatitis A and B, Diphtheria, Pertussis, and Tetanus) for the general public may have widespread health implications. 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 vaccinated may be at future risk for neurological complications, while those currently receiving injections may develop similar problems in the future. The majority of those vaccinated with the anthrax vaccine absorbed (AVA) vaccine to date have been service personnel. As serious as this may be

for the potential for adjuvant-associated complications in this population, legislation now before U.S. Congress may mandate similar vaccination regimes for the civilian population as well (Biodefense and Pandemic Vaccine and Drug Development Act of 2005). If a significant fraction of the military and civilians vaccinated develop neurological complications, the impact on U.S. society would be profound. Whether the risk of protection from a dreaded disease outweighs the risk of toxicity is a question that demands our urgent attention.

Figure 1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**
***
**
**
Control
Squalene
Aluminum
Aluminum+ Squalene
B
Open Field
Time (s) spent in outer perimeter in a 5 min session
300
200
100
0
10 15 20 25
Week
*
Control
Squalene
Aluminum
Aluminum+ Squalene
C
Water Maze
Errors/3 trials
10.0
7.5
5.0
2.5
0.0
0 A B 5 10 15 20 25
Week
*
Control
Squalene
Aluminum
Aluminum+ Squalene

Figure 2

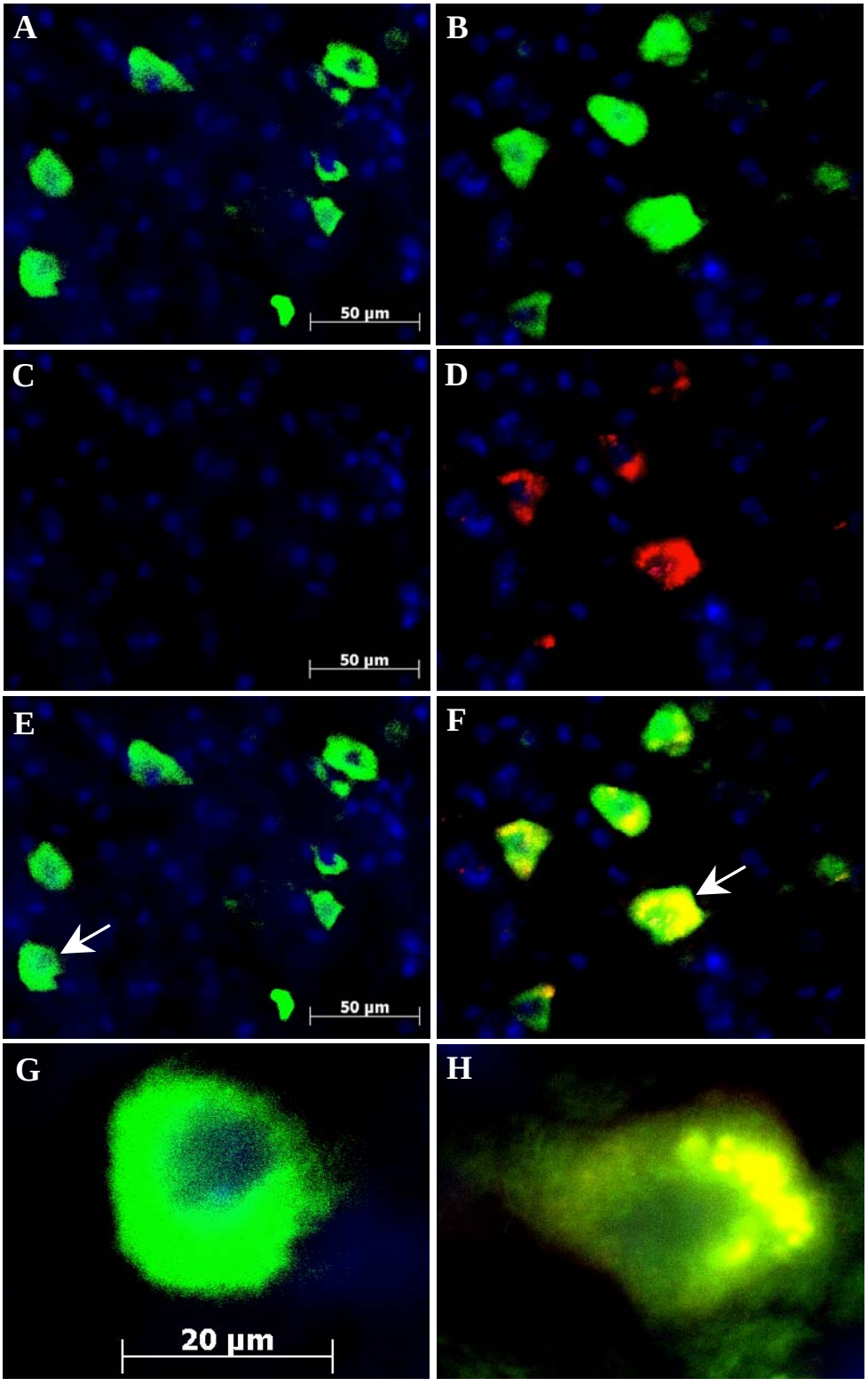

Figure 3

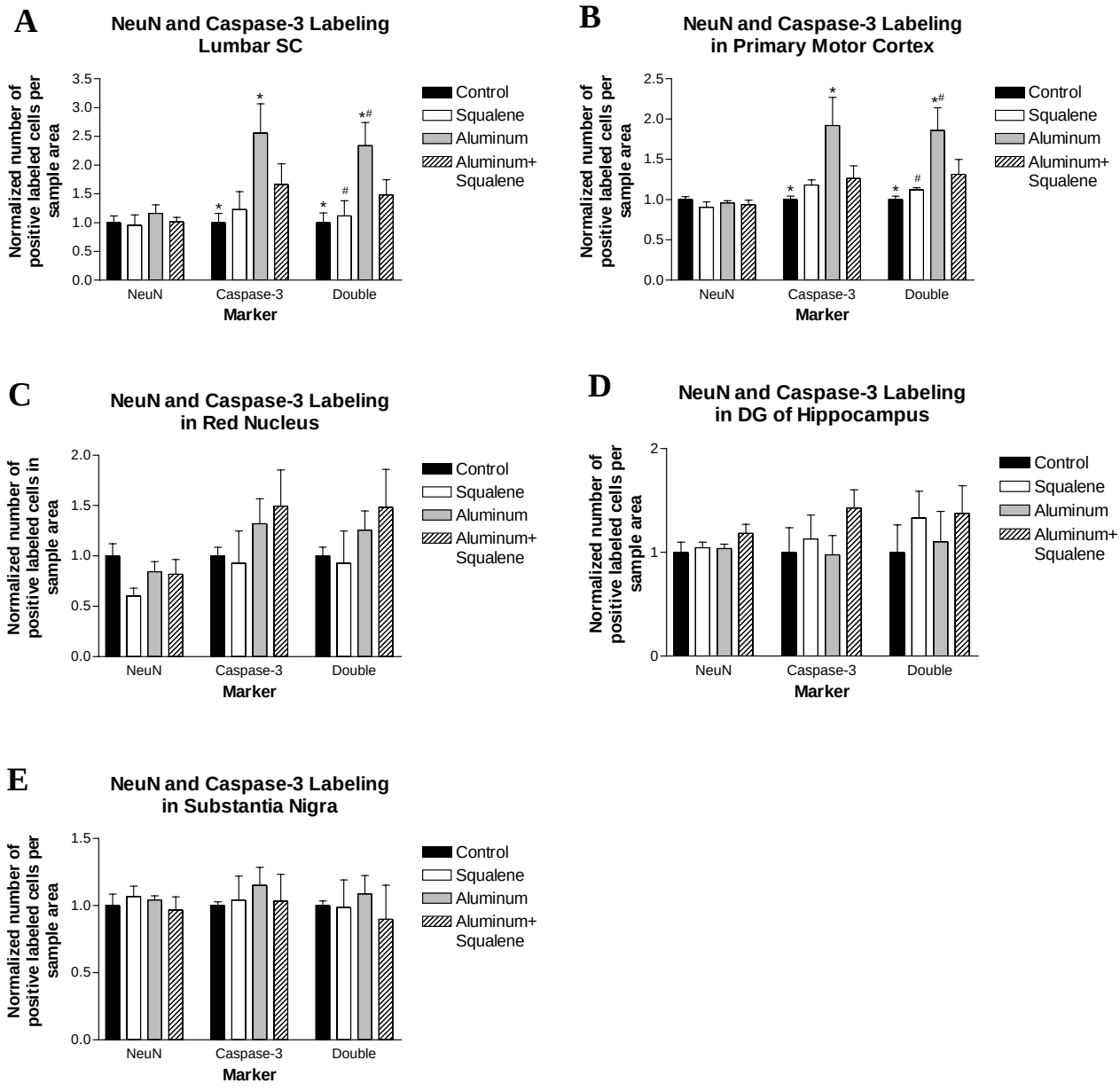
A
NeuN and Caspase-3 Labeling
Lumbar SC
Normalized number of positive labeled cells per sample area
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
NeuN
Caspase-3
Double
Marker
Control
Squalene
Aluminum
Aluminum+ Squalene
B
NeuN and Caspase-3 Labeling
in Primary Motor Cortex
Normalized number of positive labeled cells per sample area
2.5
2.0
1.5
1.0
0.5
0.0
NeuN
Caspase-3
Double
Marker
Control
Squalene
Aluminum
Aluminum+ Squalene
C
NeuN and Caspase-3 Labeling
in Red Nucleus
Normalized number of positive labeled cells in sample area
2.0
1.5
1.0
0.5
0.0
NeuN
Caspase-3
Double
Marker
Control
Squalene
Aluminum
Aluminum+ Squalene
D
NeuN and Caspase-3 Labeling
in DG of Hippocampus
Normalized number of positive labeled cells per sample area
2
1
0
NeuN
Caspase-3
Double
Marker
Control
Squalene
Aluminum
Aluminum+ Squalene
E
NeuN and Caspase-3 Labeling
in Substantia Nigra
Normalized number of positive labeled cells per sample area
1.5
1.0
0.5
0.0
NeuN
Caspase-3
Double
Marker
Control
Squalene
Aluminum
Aluminum+ Squalene

Figure 4

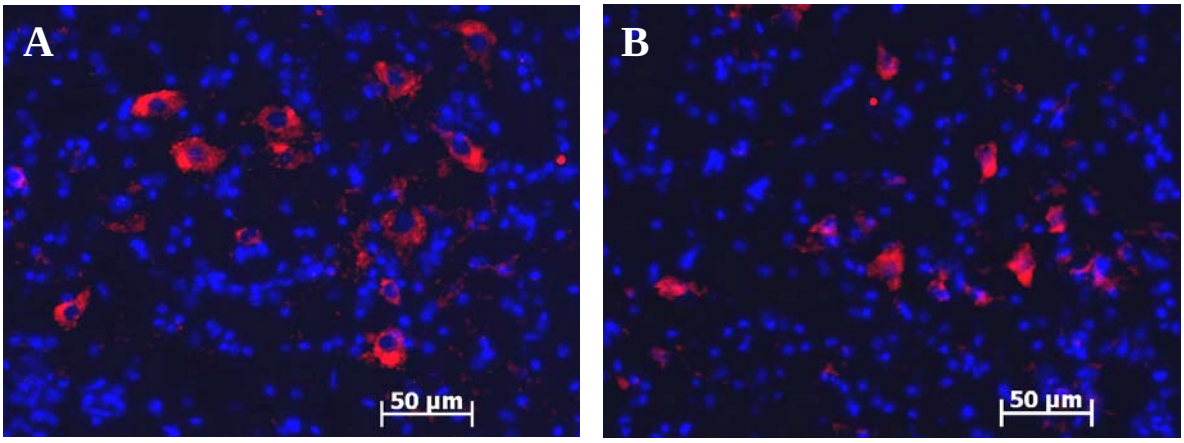

Figure 5

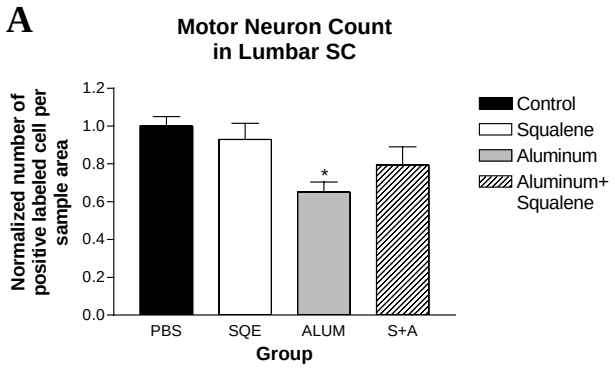
A
Motor Neuron Count
in Lumbar SC
Normalized number of positive labeled cell per sample area
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
PBS
SQE
ALUM
S+A
Group
Control
Squalene
Aluminum
Aluminum+ Squalene

Figure 6

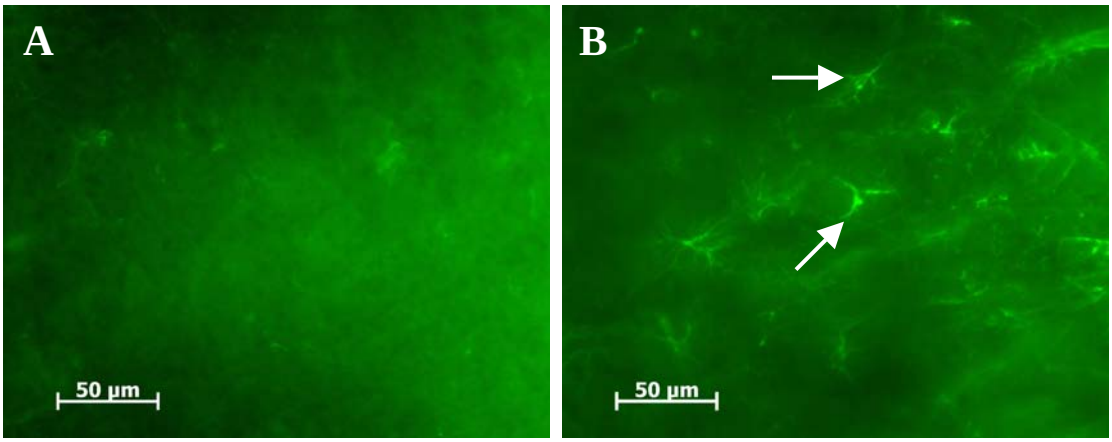

Figure 7

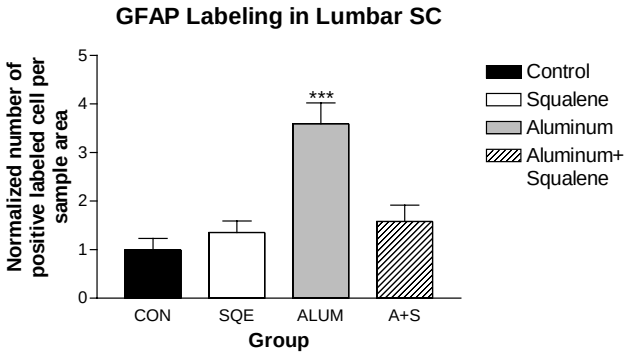
GFAP Labeling in Lumbar SC
Normalized number of positive labeled cell per sample area
5
4
3
2
1
0
***
CON
SQE
ALUM
A+S
Group
Control
Squalene
Aluminum
Aluminum+ Squalene

Table 1

Comparison of human ALS and GWS symptomology with GWS mouse model.			
Symptoms	**ALS***	**GWS†**	**GWS Mouse Model**
Muscular motor loss	✓	✓	✓
Enhanced anxiety	✓	✓	✓
Memory impairment	✓	✓	✓
Dermatitis		✓	✓

*(Bromberg, 2002). †(Haley et al., 1997).

Figure Captions

Fig. 1. Motor and cognitive effects of adjuvants. A: The wire mesh hang test measures the latency to fall when suspended from a wire mesh, as a test of muscle strength and endurance. Mice injected with aluminum hydroxide showed a significant decrease in muscular strength and endurance (-50%) compared to controls. Mice injected with squalene or both adjuvants did not show a significant decrease in muscular strength. B: Open field tests (during weeks 7-24) records the time the animal spends in the outer perimeter, as a measure of anxiety. Animals with increased anxiety will increasingly circle the perimeter. Mice injected with aluminum hydroxide show a significant increase in anxiety (+38%) compared to controls. Mice injected with squalene or both adjuvants did not show any significant effect. C: The radial arm water maze (5 arms) was used to test spatial and reference memory; animals were required to memorize and locate a rescue platform and their errors were recorded over 3 trials. 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 squalene (0.9 errors) or aluminum hydroxide (1.2 errors) did show increased errors after week 20 but these values were not significant. A=1st injection, B=2nd injection. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$, one-way ANOVA.

Fig. 2. NeuN and activated caspase-3 fluorescent labeling in ventral horn of lumbar spinal cord. Green = Neuron specific nuclear protein (NEUronal Nuclei: NeuN); Red = Activated caspase-3 antibody; specific marker for staining apoptotic cells. Yellow = Co-localization of NeuN and activated caspase-3 indicating apoptotic neuronal cell death.

Blue = Nuclear DAPI (4', 6 diamidino-2-phenylindole) counterstaining. A: Control shows NeuN labeling (Magnification: 40x). B: NeuN labeling in aluminum injected mouse. C: Control animals show no labeling of activated caspase-3 antibody. D: Animals injected with aluminum hydroxide show clear labeling of activated caspase-3 antibody. E: Tissue from control animal shows NeuN labeling but no activated caspase-3 labeling (Magnification: 40x; white arrow indicates neuron enlarged in figure 1G). G: Enlargement of neuron from figure 1E shows positive NeuN labeling with no activated caspase-3 labeling (Magnification: 100x); F: Mice injected with aluminum hydroxide show increased positive labeling of NeuN and anti-active caspase-3 compared to controls indicating apoptosis (Magnification: 40x; white arrow indicates neuron enlarged in figure 1H). H: Enlargement of neuron from 1F shows clear double labeling of NeuN and anti-active caspase-3. (Magnification: 100x). A-F: Scale bar = 50 µm. G, H: Scale bar = 20µm.

Fig. 3. A: Cell counts for NeuN and activated caspase-3 labeling in ventral horn of lumbar spinal cord. 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 (+155%) in mice injected with aluminum hydroxide compared to controls. NeuN and activated caspase-3 double labeling show significantly increased apoptotic neuronal cells (+133%) in mice injected with aluminum hydroxide compared to control and squalene injected groups. B: NeuN counts (n=20, 5 per group) show no significant difference between groups. Animals injected with aluminum hydroxide show a significant increase in

activated caspase-3 (+92%) and double labeling (+85%) in primary motor cortex compared to controls. Compared to squalene-injected mice, aluminum mice showed a significant increase (+65%) in double labeling. C: Cell counts (n=20, 5 per group) performed in the red nucleus show an increase in activated caspase-3 and double labeling in both aluminum groups compared to controls, but this was not significant. D: Hippocampal cell counts (n=20, 5 per group) performed on the polymorph layer of the dentate gyrus (DG) 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, but these results were not statistically significant. E: 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. Data are means ± S.E.M *, $^{\#}$ $p<0.05$ versus control and squalene mice, **, $p<0.01$ versus control mice using one-way ANOVA analysis.

Fig. 4. Cholinacetyltransferase (ChAT) fluorescent labeling in ventral horn of lumbar spinal cord. A: Control animal shows clear ChAT labeling and health motor neuron shape (20x magnification). B: Aluminum injected animal shows decreased ChAT labeling and abnormal morphology of motor neurons compared to controls (20x magnification). Scale bar = 50 µm.

Fig. 5. Motor neuron cell counts after ChAT fluorescent labeling in ventral horn of lumbar spinal cord. 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 ± S.E.M *** $p<0.001$ versus control mice using one-way ANOVA analysis.

Fig. 6. Glial fibrillary acidic protein (GFAP) fluorescent labeling in ventral horn of lumbar spinal cord. A: Control animal shows little GFAP labeling indicating rare presence of astrocytes (40x magnification). B: Aluminum injected animal shows increased GFAP labeling and greater number of astrocytes (white arrows) compared to controls (40x magnification). Scale bar = 50 µm.

Fig. 7. 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 when compared to controls, while mice injected with aluminum showed the greatest number of astrocytes present (approximately 3.5 times greater than controls). Data are means ± S.E.M *** $p<0.001$ versus control mice using one-way ANOVA analysis.

Table 1. Table summary of human ALS and GWS symptomology compared with GWS mouse model. This table outlines the similarities between human ALS and Gulf War

syndrome. From this table, we can see that overlapping symptoms present in ALS and some GWS patients are represented in our mouse model of GWS.

ANIMAL ETHICS COMMITTEE APPROVAL

Protocols governing the use of animals were approved by review committees of the University of British Columbia and were in compliance with guidelines published by the Canadian Council on Animal Care and are in accordance with the international guidelines including the NIH Guide for the Care and Use of Laboratory Animals, as well as the EEC Council Directive.

CONFLICT OF INTEREST STATEMENT

Petrik has not received any grants or funding from Bioport, Chiron, Corixa, nor any other pharmaceutical companies. All the other authors have viewed this article and declare that they have no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by grants from the US Army Medical Research and Materiel Command (#DAMD17-02-1-0678), Scottish Rite Charitable Foundation of Canada, and the Natural Science and Engineering Research Council of Canada (to CAS). We would like to thank Dr. Jason Wilson (University of British Columbia, B.C., Canada), Dr. Meryl Nass (Mount Desert Island Hospital, Maine, U.S.A.), and Dr. Reyniel Cruz-Aguado (University of British Columbia, B.C., Canada), for their invaluable comments and advisory contributions to this project and manuscript.

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