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ORIGINAL ARTICLE

Aluminum Adjuvant Linked to Gulf War Illness Induces Motor Neuron Death in Mice

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Abstract

Gulf War illness (GWI) affects a significant percentage of veterans of the 1991 conflict, but its origin remains unknown. Associated with some cases of GWI are increased incidences of amyotrophic lateral sclerosis and other neurological disorders. Whereas 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, an animal model for examining the potential neurological impact of aluminum hydroxide, squalene, or aluminum hydroxide combined with squalene was developed. Young, male colony CD-1 mice were injected with the adjuvants at doses equivalent to those given to US military service personnel. All mice were subjected to a battery of motor and cognitive-behavioral tests over a 6-mo period postinjections. Following sacrifice, central nervous system tissues were examined using immunohistochemistry for evidence of inflammation and cell death. Behavioral 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 wk; about 50%). Significant cognitive deficits in water-maze learning were observed in the combined aluminum and squalene group (4.3 errors per trial) compared with the controls (0.2 errors per trial) after 20 wk. Apoptotic neurons were identified in aluminum-injected animals that showed significantly increased activated caspase-3 labeling in lumbar spinal cord (255%) and primary motor cortex (192%) compared with the controls. Aluminum-treated groups also showed significant motor neuron loss (35%) and increased numbers of astrocytes (350%) in the lumbar spinal cord. 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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Index Entries: Adjuvant; ALS; aluminum hydroxide; anthrax; Gulf War illness; neurotoxicity; squalene; vaccine.

Introduction

Gulf War illness (GWI), popularly termed "Gulf War syndrome," is a spectrum of disorders among veterans of the Persian Gulf War (1990–1991) 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). Previous studies conducted on Gulf War veterans by the US Department of Defense (DOD), the US Department of Veteran Affairs, and the UK Gulf War Research Illness Unit have established a strong link between Gulf War-era service and the occurrence of GWI (Hom et al., 1997; Unwin et al., 1999; Kang et al., 2002; Wolfe et al., 2002; Dyer, 2004).

Recent studies have also established a correlation between Gulf War service and a neurological cluster of amyotrophic lateral sclerosis (ALS)–Gulf War illness (ALS–GWI; Charatan, 2002; Horner et al., 2003; Weisskopf et al., 2005). GWI can be partially described as a neurological illness that might carry an ALS component because of the overlapping symptomatology seen in ALS–GWI and classical ALS. According to a nationwide study by the Department of Veteran Affairs, deployed veterans of the Persian Gulf War are twice more likely to develop ALS than nondeployed veterans and the civilian population (Samson, 2002). Overall, GWI, however, does not appear to distinguish between troops who were deployed to the Gulf against 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). The only other known ALS cluster involves various geographical loci in the western Pacific expressing as a spectrum of neurological disorders termed ALS–parkinsonism dementia complex (Kurland, 1988; Murakami, 1999). ALS–parkinsonism dementia complex has been linked to environmental factors (Shaw and Wilson, 2003).

Both ALS clusters offer the possibility to identify causal environmental and/or genetic factors involved in sporadic ALS. Regarding 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 (Sartin, 2000; Kalra et al., 2002), organophosphates (Abou-Donia et al., 1996; Kurt, 1998), vaccines (Hotopf et al., 2000), heavy metals (Ferguson and Cassaday, 2001–2002), and bacterial infections (Taylor et al., 1997; Nicolson et al., 2002).

In recent years, increased scrutiny has focused on vaccines, in particular the anthrax vaccine absorbed (AVA; Nass, 1999), largely owing to the observation that nondeployed but vaccinated US 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 with control cohorts (Unwin et al., 1999). In contrast, Hunter et al. (2004) released a study that examined health effects of Canadian soldiers postanthrax vaccination but found no apparent link to the AVA vaccine and its adverse health effects. Notably, however, the study only monitored health outcomes for a maximum of 8-mo postvaccination; typically, patients with GWI did not express symptoms until years after the war. French soldiers participating in the war did not receive the AVA vaccine but did show some GWI-related disorders (respiratory, neurocognitive, psychological, and musculoskeletal), but no ALS symptoms were reported (Salamon et al., 2006).

The anthrax vaccine, in common with many other vaccines in wide usage, contains one chemical of particular interest from a neurological perspective: aluminum hydroxide. A second chemical, the lipid polymer squalene (a precursor to cholesterol), has been found in some lots of AVA (Plaisier, 2000); however, manufacturers of the AVA vaccine along with the 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.

Aluminum in various forms is the most common and currently licensed adjuvant and is generally regarded by industry and regulatory agencies as safe. Previous studies have found no adverse or long-term health effects (Baylor et al., 2002; Kanra et al., 2003; Jefferson et al., 2004) and the Food and Drug

Administration 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). 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, 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. Median lethal $dose_{50}$ values (for subcutaneous injection) for either aluminum hydroxide or squalene have not been published to date to the best of our knowledge (J.T. Baker Material Safety Data Sheets).

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

Given the controversies surrounding AVA and its known and suspected vaccine adjuvants, the experiments described in this article were designed in order to provide an accurate multilevel analysis of the potential impact of aluminum hydroxide and squalene on the nervous system over extended time periods in an outbred strain of young male mice. The conditions chosen in the model system were intended to mimic the administration of AVA to young, predominantly male, US and other coalition military service personnel.

Methods

Experimental Animals, Diet, and Tissue Collection

Young adult CD-1 male mice were used in the study (3 mo old and weight approx 35 g at experiment onset). Younger animals were deliberately chosen to mimic the age of service during the Gulf War (Haley, 2003). Four treatment groups were used; control (n = 10) injected with saline/phosphate-buffered saline (PBS), aluminum hydroxide (n = 11), squalene (n = 10), and aluminum hydroxide + squalene (n = 10). All animals were housed solitarily at the Jack Bell Research Center animal care facility in Vancouver, BC, Canada. An ambient temperature of 22°C and a 12/12 h light cycle were maintained throughout the experiment. All mice were fed Purina™ mouse chow *ad libitum*. Mice were subjected at regular intervals to specific behavioral tests, including wire-mesh hang (twice a week), open field (once a week), and water maze (once a week) over a period of 6-mo postinjection. The order in which the animals were tested was randomized for each trial. Mice were sacrificed with an overdose of halothane and perfused with 4% paraformadehyde. Central nervous system (CNS) tissues were collected for histological examination. Fixed brains and spinal cords from all mice were transferred to a 30% sucrose/phosphate-buffered saline (PBS) solution for overnight incubation and then frozen and stored at –80°C until sectioning. The CNS sections were cryoprotected in 30% ethylene glycol with 20% glycerol-dibasic and monobasic sodium phosphate solution and kept frozen at –20°C until use. All brain tissue blocks were mounted in Tissue-Tek optimum cutting temperature (O.C.T) compound (Sakura, Zoeterwoude, Netherlands), and then sectioned by cryostat into 30-µm coronal slices. Spinal cords were sectioned at 25 µm in the transverse plane.

Adjuvants

Alhydrogel™, an aluminum hydroxide ($Al[OH]_3$) gel suspension, was used as a source of aluminum hydroxide. Alhydrogel 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 ($C_{30}H_{50}$)-containing adjuvant was manufactured by Corixa Corporation (Seattle, WA). Both adjuvants were supplied by Sigma, Canada.

Aluminum

To calculate the approximate human dosages of aluminum hydroxide and squalene for the experiments the following information was used. The AVA vaccine for human use is made by Bioport Corporation, Lansing, MI. According to product data sheets from the Michigan Biologic Products

Institute (MBPI, Lansing, MI; Bioport's predecessor) a single dose of AVA vaccine contains 2.4 mg of aluminum hydroxide (equivalent to 0.83 mg of aluminum). Based on an average human body weight of 70–80 kg, the amount per kilogram body weight is approx 30–34 µg/kg. Soldiers or civilians receiving the vaccine would have received between 30 and 34 µg/kg (one injection) up to 120–136 µg/kg if four injections were received.

Squalene

As noted earlier, both Bioport Corporation (Lansing, MI) and the MBPI deny the addition of squalene in AVA formulation. Therefore, MF59 was calculated based on current vaccines in use outside the United States that employs a squalene-containing adjuvant oil emulsion. This adjuvant in experimental influenza vaccines (Chiron Corporation Emeryville, CA) uses a concentration of 5% squalene. Based on the total volume of the MF59 injection (0.5 mL), this would be equivalent to 0.025 mL of squalene. Again, based on an average 70–80 kg human, the amount per injection would be approx 0.31–0.35 µg/kg for one injection, as much as 1.24–1.40 µg/kg for a full series of four injections. The adjuvant injections in the mice were calibrated based on average animal weight for 3-mo-old male CD-1 mice (approx 35 g). Performing two injections as an average (range 1–4) based on US DOD usage during the Gulf War in 1991 was chosen. Based on the human values cited earlier, mice receiving aluminum hydroxide received two doses of 50 µg/kg (suspension) in a total volume of 200-µL sterile PBS (0.9%). The mice in this experiment would, therefore, have received 100 µg/kg against a probable 68 µg/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 µg/kg over two injections compared with the likely human dose of 0.62–0.71 µg/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-µL PBS.

Immunization

The injection site for human administration is typically subcutaneous over the deltoid muscle. For injections in mice, a subcutaneous injection into the loose skin behind the neck (the "scruff") was used for ease of injection and to minimize discomfort. Animals received two injections (2 wk apart) of aluminum hydroxide, squalene, aluminum hydroxide + squalene, or PBS. This immunization protocol mimicked the anthrax vaccine dose schedule set by the Anthrax Vaccine Immunization Program except for the route of administration.

Behavioral Tests

In all behavioral tests and histological assays, the experimenters were blind to the identity of treatment groups of the animals or samples.

Wire-Mesh Hang

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

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 approx 5-cm thick. An overhead video camera was used to record mouse locomotion. The number of squares crossed in a measured area (outside, inside, and center perimeters) over a 5-min period was counted. 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 into the center of the arena (Crawley et al., 1997). Testing was conducted once a week for the duration of the experiment.

Water Maze

The water maze was used to evaluate spatial and reference memory, both forms of long-term memory (Morris, 1984). The water-maze setup included a pool, 1.3 m in diameter (Everts and Koolhaas, 1999), five radial arms 30-cm high, and a rescue platform 5 mm above the water level. The mice were trained for 4 d, at three trials per day before 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 60 s, after which they were guided to the platform using a ruler. At 90 s, the handler placed mice on the platform if they still had not reached it on their own. Training was terminated when the mice consistently found the platform within 25 s on four 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.

Immunohistochemistry

Neuronal Nuclei and Activated Caspase-3 Labeling

Mouse neuronal nuclei (NeuN) antibody (Chemicon International, Temecula, CA, 1:300) a DNA-binding and neuron-specific nuclear protein was used to identify neurons (Mullen et al., 1992; Wolf et al., 1996). Mounted sections were rinsed in 10% Tris-ethylene diamine tetraacetic acid (EDTA) buffer and microwaved for 10 min. After heating, sections were allowed to cool for 20 min and were then incubated in working solution of mouse on mouse (MOM™) immunoglobulin (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 immunoglobulin (Ig)G reagent for 10 min and incubated with fluorescein-avidin DCS for 5 min, then blocked with 10% NGS for 1 h. Sections were incubated with rabbit-antiactivated caspase-3 antibody (Promega; Madison, WI, 1:250) for 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 because of having the use of Vector MOM kit for NeuN. All steps were performed at room temperature unless specified otherwise.

Choline Acetyltransferase Labeling

Choline acetyltransferase (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 (PBST) twice for 15 min. Sections were then blocked in 5% normal goat serum (NGS) with 5% bovine serum albumin (BSA) for 3 h, 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 antigoat IgG antibody (DuoLuX™, Elite ABC Kit, Vector Laboratories; 1:200) at room temperature and mounted with fluorescent DAPI.

Glial Fibrillary Acidic Protein Labeling

Glial fibrillary acidic protein (GFAP) is a member of the class III intermediate filament protein family and stains reactive rodent and normal human brain astrocytes as well as those induced by a variety of CNS injuries (Lee et al., 1984; Tohyama et al., 1991). Antiglial 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 slide-mounted sections and pretreated in PBST twice for 5 min. Sections were then blocked in 10% NGS + 1% BSA in PBST for 2 h, then incubated with primary antibody rat anti-GFAP (in PBST with 1% NGS + 1% BSA) at 10 µg/mL (1:100) in a humidified chamber at room temperature (23°C) overnight. Sections were then incubated for 1 h in anti-rat fluorescein isothiocyanate antibody (1:200 dilutions in PBS, Serotec Laboratories, Raleigh, NC) incubate for at room temperature and mounted with fluorescent DAPI.

Microscopy

Brain and spinal cord sections processed with fluorescent materials were viewed with a Zeiss Axiovert (Carl Zeiss Canada Ltd., Toronto, ON) microscope zoom at ×40 and ×100 (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; fluorescein isothiocyanate antibody was viewed with a 490,494/520,525 nm filter. Images were captured using AxioVision 4.3 software.

Histological Measurements

Neuronal Nuclei and Active Caspase-3

Multiple brain (n = 3) and lumbar spinal cord (n = 8) 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. Regions of interest (ROI) were defined using landmarks from mouse brain and spinal cord stereotaxic atlases (Sidman et al., 1971; Paxinos and Franklin, 2001). All sections were counted in an unbiased manner. Cell counts included the total number of cells labeled with either NeuN, activated caspase-3, or both (double labeling) counted under a ×40 objective lens.

Choline Acetyltransferase

Lumbar spinal cord sections (n = 8) from each mouse were captured and ROIs defined using the methods described earlier. Eight mice from each treatment group were used for the assay of lumbar spinal cord. Ventral root motor neurons were counted under a ×40 objective lens. All motor neurons in the field of view were counted.

Glial Fibrillary Acidic Protein

Lumbar spinal cord sections (n = 8) from each mouse were captured and ROIs defined as mentioned earlier. Eight mice from each treatment group were used for the assay of lumbar spinal cord. Counts were conducted under a ×40 objective lens, including all astrocytic cells in the field of view.

Squalene Antibody Assay

Serum was collected from animals through tail bleed and sent to Tulane University Health Sciences Center for Analysis. Squalene was diluted 10–10^4-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 before the addition of 5 µL of mouse serum samples (1:100–400 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 antimouse IgG (Sigma, St Louis, Mo), diluted 1:1000, was added. After 60 min incubation, the strips were again washed as above, and 2 mL of blocking buffer containing avidin-conjugated horseradish peroxidase (Jackson Immuno Research, West Grove, PA), diluted 1:500, was added. Following another 60 min incubation, the strips were washed and 2-mL 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, then qualitatively scored on a scale of 0–4 (*see* Asa et al., 2002).

Statistics

Values for each mouse on the individual tasks and in the cell counts were used to calculate mean ± S.E.M. for each group and condition. 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

Behavioral Effects

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 with the controls (100% for all data; Fig. 1A). Squalene-injected mice

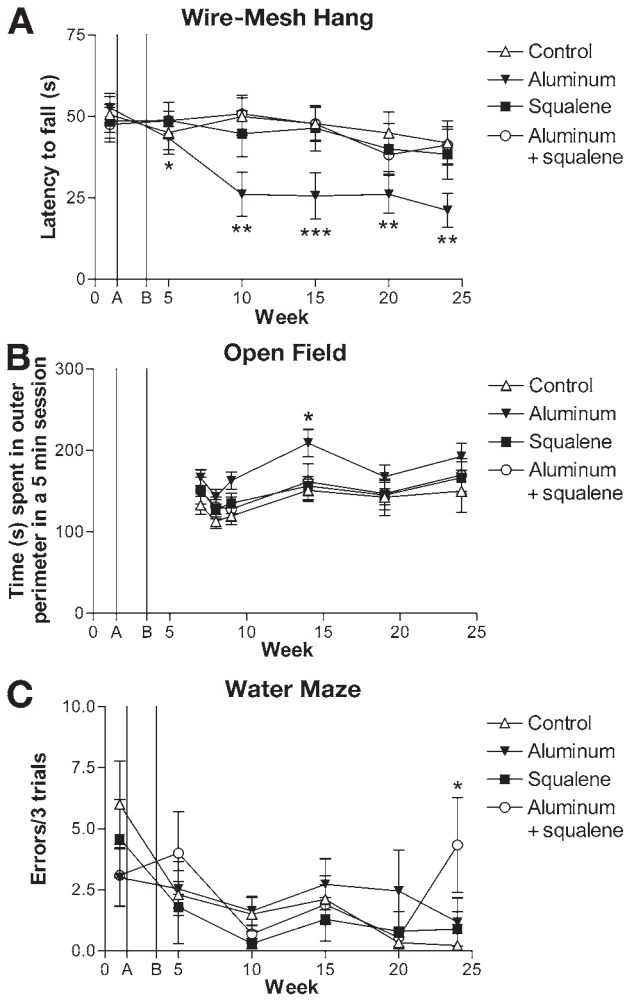


Fig. 1. Motor and cognitive effects of known and presumed AVA adjuvants. **(A)** Wire-mesh hang test. Mice injected with aluminum hydroxide showed a significant decrease in muscular strength and endurance (50%) compared with the controls (100%). Mice injected with squalene or both adjuvants did not show a significant decrease in muscular strength. **(B)** Open-field tests (during weeks 7–24). Mice injected with aluminum hydroxide show a significant increase in anxiety (138%) compared with the controls. Mice injected with squalene or both adjuvants did not show any significant effect. **(C)** The radial arm water maze (five arms). Mice injected with aluminum hydroxide (1.2 errors) or squalene (0.9 errors) did show increased errors after week 20 but these values did not reach statistical significance. Mice injected with both adjuvants showed a significant increase in errors after week 20 (4.3 errors), whereas, controls achieved 0.2 errors. A = first injection, B = second injection. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$; one-way ANOVA.

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.

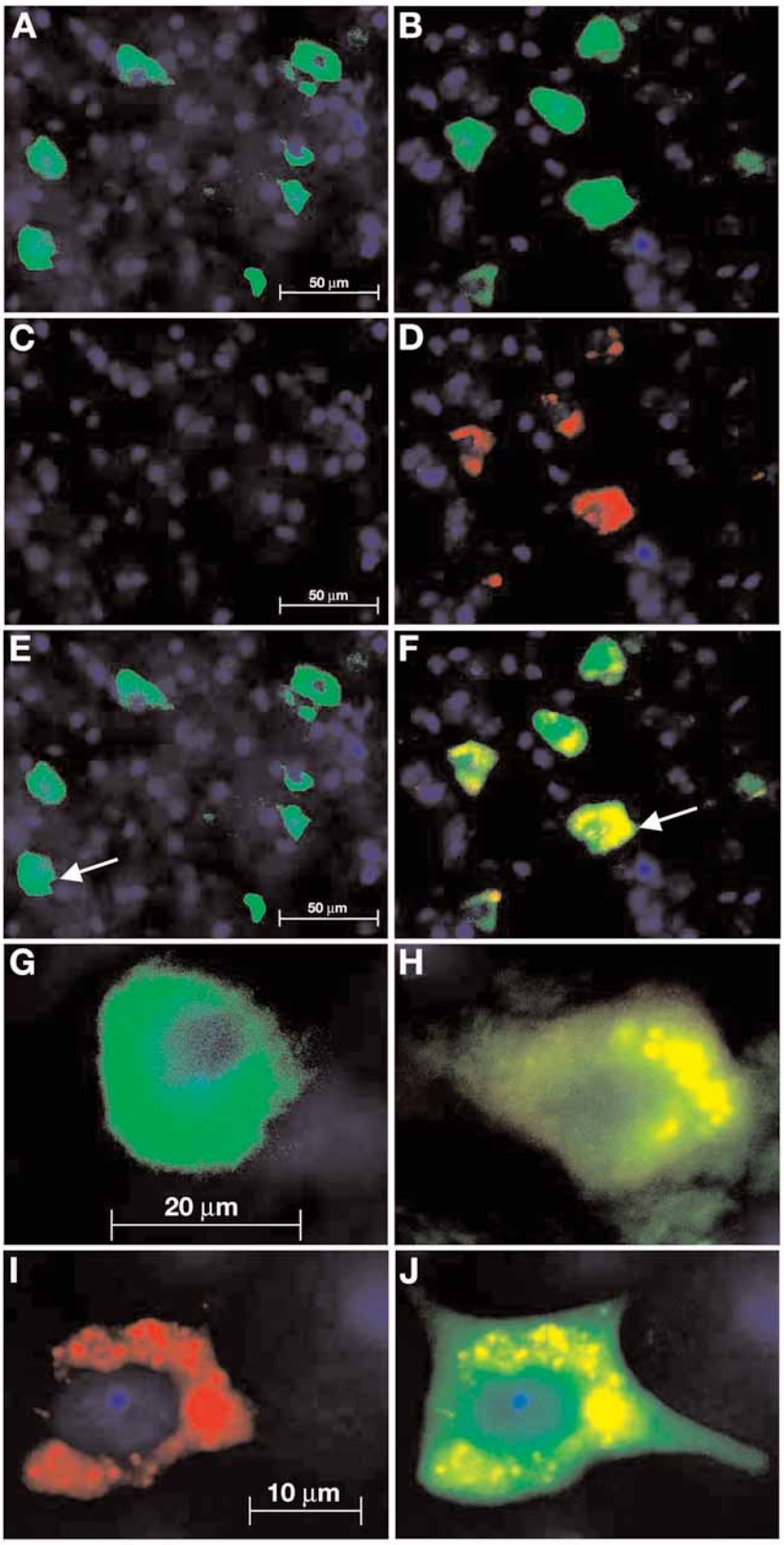
Aluminum-injected mice showed a significant increase in anxiety levels at week 14 (138%) as measured by the longer time spent in the outer perimeter during the open-field tests (Fig. 1B). After 14 wk, the aluminum group continued to show increased levels of anxiety compared with the controls but these 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.

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 with the controls (0.2 errors; Fig. 1C).

CNS Pathology

Mice injected with PBS showed little or no activated caspase-3 labeling in ventral lumbar spinal cord (Figs. 2C,E,G and 3A). 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 (Figs. 2D,F,H–J and 3A). 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 addition to the spinal cord, other brain structures involved in motor function were also examined. NeuN and activated caspase-3 immunohistology was performed on the primary motor cortex, the red nucleus, substantia nigra, and hippocampus because these areas are affected in the human motor diseases such as ALS and Parkinson's disease (Sasaki et al., 1992; Eisen and Weber, 2001; Tsuchiya et al., 2002). Quantitative analysis of NeuN labeling showed comparable numbers of labeled neurons in all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treatment groups (Fig. 3A–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 with the controls (Fig. 3B). 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. 3C). Analysis of the substantia nigra region did not reveal any differences in labeling between groups (Fig. 3D). In the hippocampus, cell counts conducted on the polymorphic layer of the dentate gyrus showed an increase in double labeling for squalene and combined groups but it did not reach statistical significance (Fig. 3E).

Only cells labeled with ChAT were included in the motor neuron counts of lumbar spinal cord. Aluminum-injected mice showed a significant reduction in motor neurons (35%) compared with the controls (Fig. 4A–C). The squalene and combined group also showed a reduction in motor neuron number that did not achieve statistical significance.

The aluminum-injected group showed a highly significant increase in the expression of GFAP-positive astrocytes (350%) greater than the controls (Fig. 5A–D). Animals treated with squalene or aluminum with squalene showed small increases in the number of astrocytes present when compared with the controls, but these differences were not statistically significant.

Squalene-Antibodies Assay

Two out of ten control animals showed the presence of squalene antibodies (SA) in the first serum specimen taken at 4 wk (2 wk postsecond 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. Three out of the eleven 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 wk).

Non-CNS Features

In addition to behavioral changes and CNS pathology, various physiological changes were observed. Hair loss at the injection site (0.5–1.0-cm 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 behavioral side-effects.

The present results indicate that anthrax vaccine adjuvants mimicking a minimal AVA administration regime (two injections) resulted in some neuropathological outcomes postinjection (Nass, personal communication). Aluminum hydroxide induced both behavioral and motor deficits, and the increased presence of apoptotic neurons and in various regions of

Fig. 2. *(Opposite page)* NeuN and activated caspase-3 fluorescent labeling in ventral horn of lumbar spinal cord. Green = NeuN; red = activated caspase-3; yellow = colocalization of NeuN and activated caspase-3; blue = nuclear DAPI. **(A,B)** NeuN labeling in control and aluminum hydroxide injected mouse lumbar spinal cord sections, respectively. **(C,D)** Control and aluminum hydroxide mouse lumbar spinal cord sections labeled with caspase-3. **(E,F)** Merge of NeuN and caspase. Magnification ×40 A–F. White arrow indicates neuron enlarged in **(G,H)**. Enlargement of neurons E,F at ×100 magnification. **(I,J)** Enlargement of another activated caspase-3 positive motor neuron at ×100 magnification. J, Merged image of activated caspase-3 and NeuN. A–F; Scale bar = 50 μm. G,H; Scale bar = 20 μm. I,J, Scale bar = 10 μm.

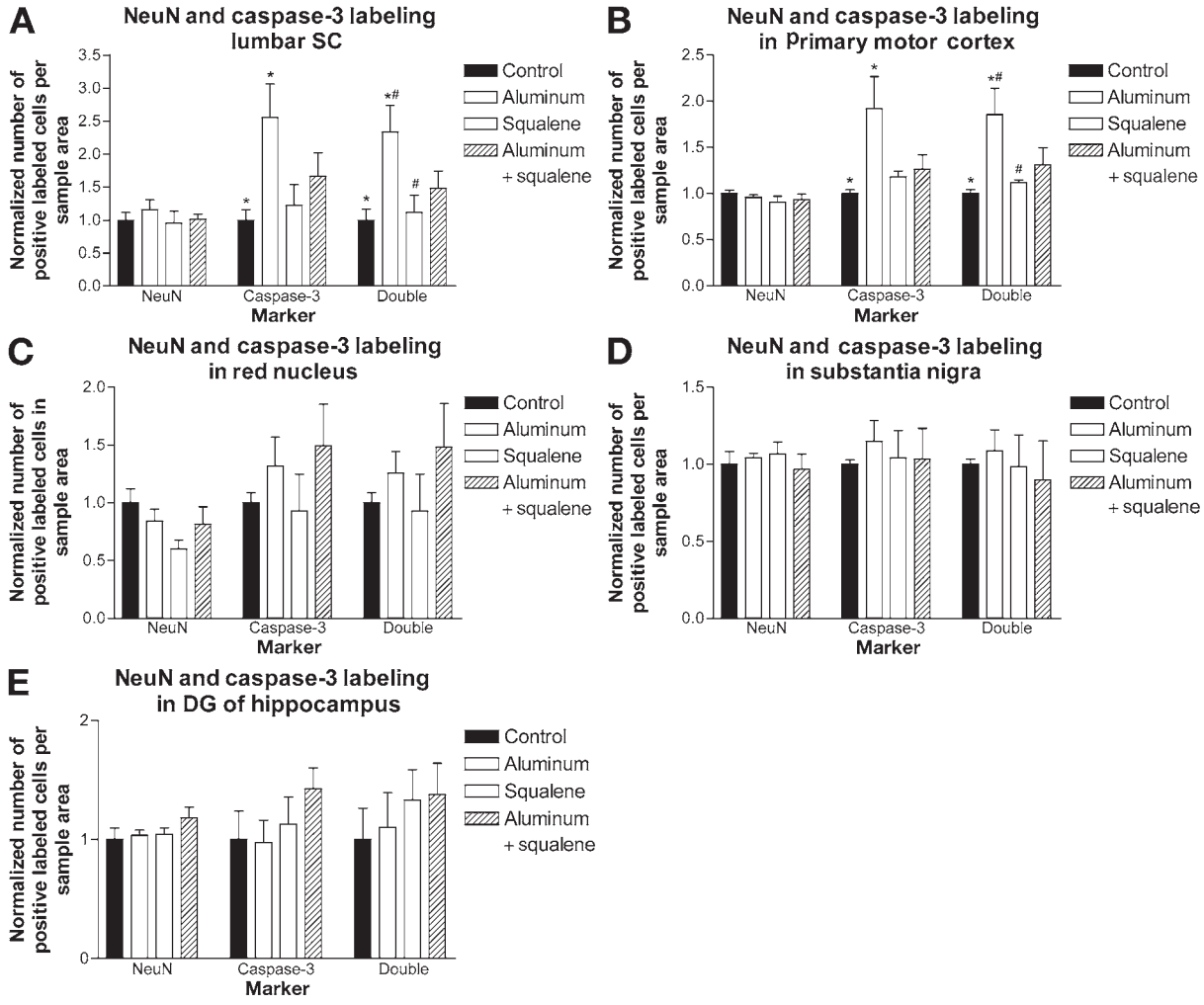

Fig. 3. **(A)** Cell counts for NeuN and activated caspase-3 labeling in ventral horn of lumbar spinal cord. NeuN counts between groups ($n = 32$, eight per group) show no significant differences indicating similar numbers of neuronal cells labeled in all groups. Activated caspase-3 marker shows significantly increased positive capsase-3 labeling (255%) in mice injected with aluminum hydroxide compared with the controls. NeuN and activated caspase-3 double labeling show significantly increased apoptotic neuronal cells (233%) in mice injected with aluminum hydroxide compared with the control and squalene injected groups. **(B)** NeuN counts ($n = 20$, five 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 with the controls. Aluminum hydroxide-injected mice showed a significant increase (165%) in double labeling when compared with the squalene-injected mice. **(C)** Cell counts ($n = 20$, five per group) performed in the red nucleus show a non significant increase in activated caspase-3 and double labeling in both aluminum groups compared with the controls. **(D)** SNpc; there was no significant difference in cell counts ($n = 20$, five per group) of NeuN and activated caspase-3 labeling between groups in the substantia nigra region. **(E)** Hippocampal cell counts ($n = 20$, five per group) performed on the polymorphic layer of the dentate gyrus show increased activated caspase-3 and double labeling in the squalene group, whereas, the combined group showed the greatest activated caspase-3 and double labeling. These results were not statistically significant. Histograms show means ± S.E.M $^{*,\#}p < 0.05$ vs control and squalene mice, $^{**}p < 0.01$ vs control mice using one-way ANOVA.

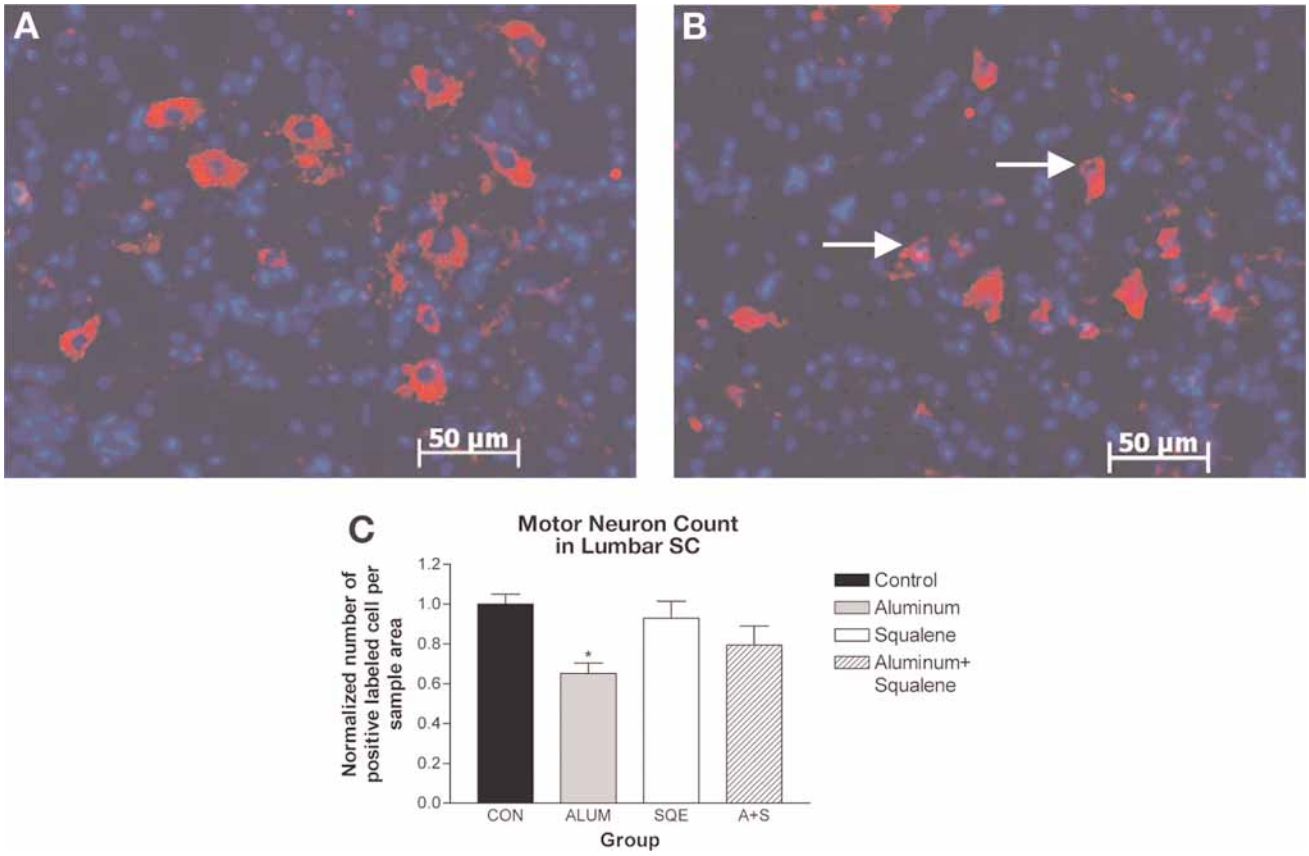

Fig. 4. Choline acetyltransferase (ChAT) fluorescent labeling in ventral horn of lumbar spinal cord. **(A)** Control section shows ChAT labeling of motor neurons (×20 magnification). **(B)** Aluminum-injected animal shows decreased ChAT labeling and abnormal morphology of motor neurons (white arrows) compared with the controls (×20 magnification). Scale bar = 50 µm. **(C)** Only cells positively labeled with ChAT were counted as motor neurons (n = 32, eight per group). Mice injected with aluminum hydroxide showed a statistically significant decrease in motor neuron number (35%) compared with the controls. There was no significant difference in motor neuron counts between all other groups compared with the controls. Data are means ± S.E.M ***$p < 0.05$ vs control mice using one-way ANOVA.

CNS with significant motor neuron loss in the lumbar spinal cord. The presence of caspase-3 labeling in cells not labeled with NeuN suggests that non-neural cells also undergo apoptosis under these conditions.

These results are consistent with a potential role for aluminum in motor neuron death in ALS. In those CNS areas tested to date (spinal cord), reactive astrocytes were present in significant numbers, indicating an inflammatory response. Previous studies have shown the increased presence of reactive astrocytes in human ALS and animal models of the disease (Nagy et al., 1994; O'Reilly et al., 1995; Levine et al., 1999; Barbeito et al., 2004).

The squalene adjuvant alone produced a small change in locomotion and anxiety testing, but the differences in the cell counts of this group with respect to controls were not significant in any CNS region. The combination of both the adjuvants showed a significant long-term memory deficit with some indications of neuronal apoptosis in the red nucleus and DG region of the hippocampus. Thus, while squalene does not appear to have the same overall impact as aluminum at sacrifice, the change in cognitive function might suggest that possible longer-term squalene effects should be examined in future studies. Regarding to the SA

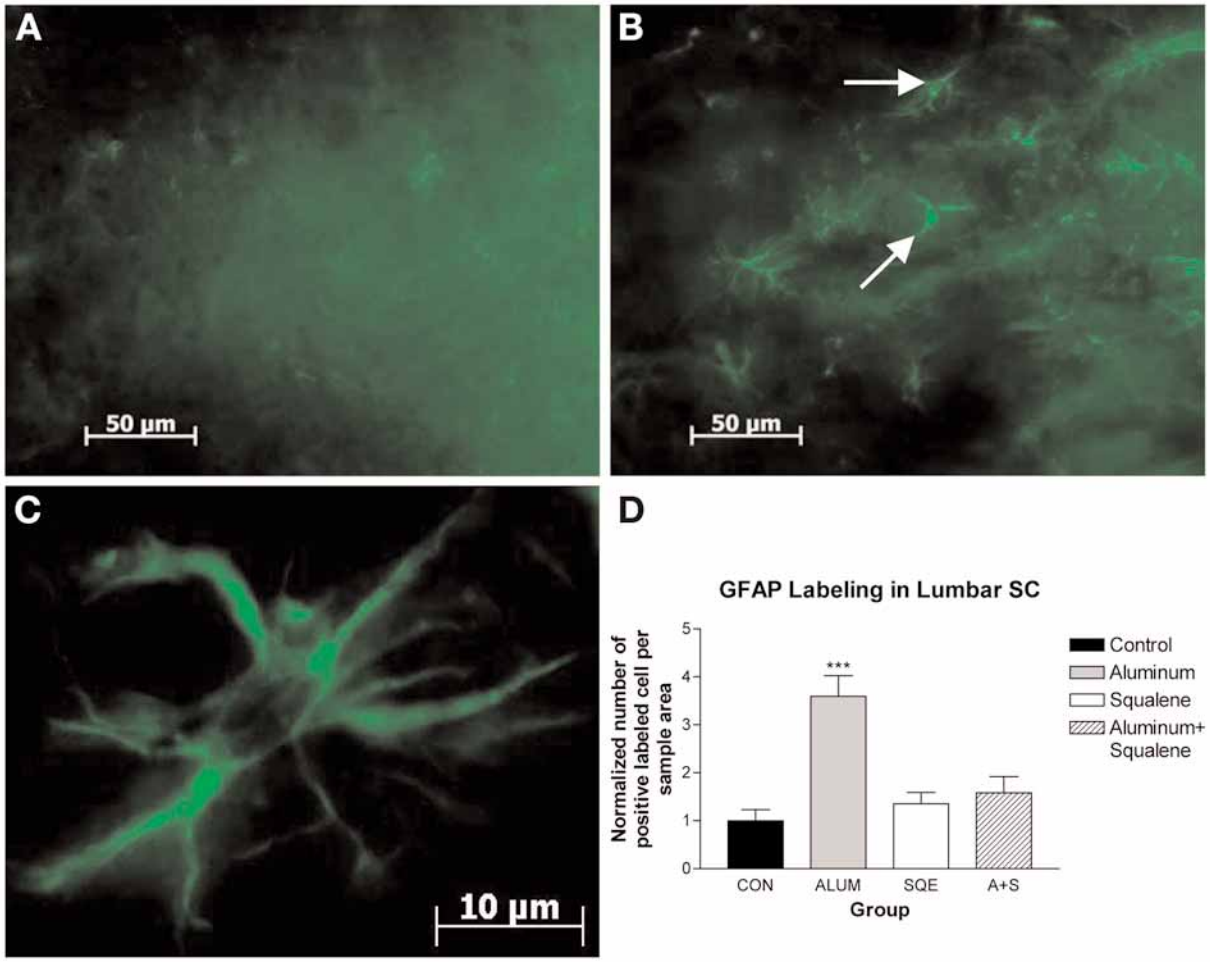

Fig. 5. GFAP-fluorescent labeling in ventral horn of lumbar spinal cord. **(A)** Control sections show little GFAP labeling. **(B)** Sections from mice injected with aluminum hydroxide show increased GFAP labeling and greater number of astrocytes (white arrows) compared with the controls (A,B ×40 magnification). Scale bar = 50 µm. **(C)** Astrocyte from aluminum injected mouse observed under ×100 magnification. Scale bar = 10 µm. **(D)** Normalized cell counts for GFAP-labeling of astrocytes in ventral horn of lumbar spinal cord (n = 32, eight per group). Squalene treated animals show a small increase in GFAP-labeled astrocytes when compared with the controls. Animals treated with both aluminum hydroxide and squalene showed a larger increase in astrocyte cell number whereas mice injected with aluminum showed the greatest increase in GFAP-labeled astrocytes (350%). Data are means ± S.E.M ***$p < 0.001$ vs control mice using one-way ANOVA.

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 the controls showed some SA-positive mice. Previous studies have suggested that naturally occurring antibodies against 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 approx 12% of animals, a number qualitatively similar to the control and aluminum hydroxide injected CD-1 mice. The relatively low incidence of SA in squalene injected mice might reflect a transient antibody production. Future experiments with more specific antibodies may resolve this issue.

Aluminum can access CNS following injections with aluminum-adjuvanted vaccines (Wen and Wisniewski, 1985; Redhead et al., 1992; Sahin et al.,

1994). Various studies have clearly demonstrated that aluminum can be neurotoxic (Crapper et al., 1973; Banks and Kastin, 1989; Joshi, 1990; Kawahara et al., 2001). For example, aluminum-injected animals show severe anterograde degeneration of cholinergic terminals in cortex and hippocampus (Platt et al., 2001). Potential toxic mechanisms of action include interference with cholinergic projections, blockage of synaptic transmission, defective phosphorylation—dephosphorylation reactions, altered rate of transmembrane diffusion and selective changes in saturable transport systems in the blood–brain barrier (BBB), reduced glucose utilization, and site-specific damage inflicted by free radicals produced by altered iron metabolism. Aluminum has also been proposed as a factor in neurodegenerative diseases based on its demonstrated neurotoxic potential 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).

Squalene has been shown to induce antibodies associated with lupus (Satoh et al., 2003) and to trigger chronic T-cell-mediated rheumatoid arthritis (Carlson et al., 2000). Its actions 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).

In addition to direct toxic actions on the CNS, aluminum, and squalene might act indirectly by stimulation of a generalized immune response. In fact, this is, what the adjuvants are placed in vaccines to do in the first place. Another possibility is that 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 Th2 to Th1 (T-helper cell type-1) immunity (Rook and Zumla, 1997, 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-based immune response (Flick-Smith et al., 2005). Such a Th1–Th2 shift could stimulate autoimmune processes that target the neurons. Whereas a plausible mechanism, a recent study of blood samples from Gulf war veterans showed evidence for Th1 immune activation (Skowera et al., 2004).

Table 1
Summary of Human ALS and GWI Symptoms Compared With the Symptoms Observed in Aluminum-Injected Mice

Comparison of human ALS and GWI symptomology with symptoms observed in aluminum-injected mice

Symptoms	ALS[a]	GWI[b]	Aluminum-injected mice
Muscular strength and endurance loss	+	+	+
Enhanced anxiety	+	+	+
Memory impairment	+	+	+
Dermatitis	–	+	+

This table also outlines the similarities between human ALS and Gulf War illness.

[a]Bromberg (2002); [b]Haley et al. (1997).

Whereas significant behavioral 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 was demonstrated. Table 1 shows a summary of human ALS and GWI symptoms compared with the symptoms 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 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 might play a crucial role. Regarding to this last point, gene–toxin interactions remain a largely unexplored area in GWI and neurological disease in general.

However, interactions of various stessors or adjuvants does 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 over ruled and deserves further investigation.

The current DOD immunization schedule requires a higher number of injections (six) than used in 1990–1991. The majority of those vaccinated with the AVA vaccine to date have been service personnel. As serious as this might be for the potential for adjuvant-associated complications in this population, legislation now before US Congress might 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, then the impact on US society would be profound.

In addition, the continued use of aluminum adjuvants in various vaccines (i.e., Hepatitis A and B, DPT, and so on) for the general public may have even more 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 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 urgent attention.

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

None of the authors have received any grants or funding from Bioport, Chiron, and Corixa, nor any other pharmaceutical companies named in this article.

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