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Cytotoxicity of immunoglobulins from amyotrophic lateral sclerosis patients on a hybrid motoneuron cell line

(autoimmune/calcium channel/cell death)

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ABSTRACT **Patients with amyotrophic lateral sclerosis possess antibodies (ALS IgGs) that bind to L-type skeletal muscle voltage-gated calcium channels (VGCCs) and inhibit L-type calcium current. To determine whether interaction of ALS IgGs with neuronal VGCCs might influence motoneuron survival, we used a motoneuron–neuroblastoma hybrid (VSC 4.1) cell line expressing binding sites for inhibitors of L-, N-, and P-type VGCCs. Using direct viable cell counts, quantitation of propidium iodide- and fluorescein diacetate-labeled cells, and lactate dehydrogenase release to assess cell survival, we document that ALS IgG kills 40–70% of cAMP-differentiated VSC 4.1 cells within 2 days. ALS IgG-mediated cytotoxicity is dependent on extracellular calcium and is prevented by peptide antagonists of N- or P-type VGCCs but not by dihydropyridine modulators of L-type VGCCs. Preincubating IgG with purified intact L-type VGCC or with isolated VGCC α_1 subunit also blocks ALS IgG-mediated cytotoxicity. These results suggest that ALS IgG may directly lead to motoneuron cell death by a mechanism requiring extracellular calcium and mediated by neuronal-type calcium channels.**

Amyotrophic lateral sclerosis (ALS) is a catastrophic human neurodegenerative syndrome that compromises upper and lower motoneuron function, leading to progressive paralysis and death. While pathogenesis of ALS is unknown, recent research has provided significant clues toward understanding the causes of selective neuronal death in this syndrome. Familial ALS (which affects 10% of ALS patients) is linked in some kindreds to mutations in Cu^{2+}/Zn^{2+}-dependent superoxide dismutase, thereby suggesting the potential role of free radicals in motoneuron injury (1). In patients with sporadic ALS, motoneuron death is hypothesized to result either from activation of ligand-gated ion channels and excitotoxicity (2–5) or from autoimmunity and antibody interaction with voltage-gated calcium channels (VGCCs) (4, 6–10). We have previously documented the presence of IgG-type antibodies to VGCCs in sporadic but not familial ALS (6). These ALS IgGs inhibit skeletal muscle L-type voltage-gated calcium currents (7–9). But while VGCCs have been implicated in regulating intracellular calcium homeostasis and neuronal survival (11–13), mitochondrial activity (14), oxidative injury (15, 16), and excitotoxic ligand-gated ion channel function (17), such effects usually result from VGCC activation. The suggestion that neuronal VGCC activation and associated increased motoneuron calcium entry might result from interaction with IgG purified from patients with ALS (ALS IgG) came from passive transfer experiments in mice, where ALS IgG selectively increased acetylcholine release from motoneuron terminals (18).

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To establish whether ALS IgG could augment neuronal VGCC-mediated calcium currents, and to determine whether resulting changes in calcium homeostasis could affect cell survival, a motoneuron–neuroblastoma hybrid cell line (VSC 4.1) was developed in our laboratory. Like mammalian motoneurons (19–22), these cells contain specific antagonist binding sites for L-type VGCCs (dihydropyridines), N-type VGCCs (ω-conotoxin), and P-type VGCCs (agatoxin IVa). While not a homogeneous clonal line, VSC 4.1 cells possess many other properties similar to both rat motoneurons and other motoneuron hybrids previously developed in other laboratories (23, 24), including dibutyryl cAMP- or 8-bromo-cAMP-inducible choline acetyltransferase, neuron-specific enolase, immunoreactive 200-kDa neurofilament protein, and synaptophysin. In this manuscript, we present evidence that ALS IgGs are cytotoxic for VSC 4.1 cells differentiated with cAMP. Such cytotoxicity is calcium dependent and mediated by neuronal (N- and P-type) VGCCs.

MATERIALS AND METHODS

Cell Culture. Parental embryonic day 15 rat ventral spinal neuron preparation, mouse N18TG2 neuroblastoma culture, somatic cell fusion, and hybrid selection were performed as described (25, 26). VSC 4.1 hybrid cells were maintained in logarithmic-phase growth on poly(L-ornithine)-precoated T-75 flasks (Corning) in Dulbecco's modified Eagle's medium/F-12 growth medium (GIBCO) containing Sato's components (Sigma) and 2% heat inactivated newborn calf serum (HyClone).

VSC 4.1 cells undergoing cAMP-induced differentiation were plated at $1.2–1.5 \times 10^6$ cells per 75-mm^2 flask and treated for 7 days with 1 mM dibutyryl cAMP or 1 mM 8-bromo-cAMP before use. Aphidicolin (0.4 μg/ml) (27) (Sigma) was added for 2 days after 24 hr in cAMP, and after 2 additional days of aphidicolin-free growth surviving cells were maintained in medium containing 1 mM cAMP and 0.025–0.05 μg of aphidicolin per ml. Differentiated hybrids were harvested by trituration after 1-hr incubation at 37°C in Ca^{2+}/Mg^{2+}-free Hanks' balanced salt solution containing 1 mM EDTA and transferred into 24-well plates (Corning) at 2.0×10^4 cells per well [for direct cell count and lactate dehydrogenase (LDH) assays] or per 11-mm-diameter glass coverslip (for fluorescence assays). Pharmacologic agents and immunoglobulins were added to cells 24 hr after replating and were maintained in growth medium for the entire exper-

Abbreviations: ALS, amyotrophic lateral sclerosis; LEMS, Lambert–Eaton myasthenic syndrome; VGCC, voltage-gated calcium channel; FDA, fluorescein diacetate; PI, propidium iodide; LDH, lactate dehydrogenase; AMPA, DL-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione.

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imental time course (unless otherwise indicated) by refeeding with 50% exchange of medium and chemicals/IgG every 2 days. Nifedipine, Bay K8644, DL-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate, and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) were purchased from Research Biochemicals (Natick, MA). ω-Conotoxin and Aga-IVa toxin were purchased from Peninsula Laboratories, while EGTA, 8-bromo-cAMP, and dibutyryl cAMP were obtained from Sigma. All ligand and voltage-gated calcium channel antagonists were tested for direct toxicity by assay *in vitro* with VSC 4.1 cells (see below), and nontoxic concentrations were then assayed for effects on ALS IgG-mediated cytotoxicity.

Survival Assays. Surviving cell number on each well or coverslip was assayed at selected times after addition of immunoglobulins or pharmacologic agents by serial quantitation of bright-phase cell profiles retained on dish substrata in defined 1-mm² fields. To verify that such counts reflected cytolysis, differentiated VSC 4.1 cells were initially counted and then treated by replacement of growth medium with phosphate-buffered saline (PBS; pH 7.4) containing 10 μM fluorescein diacetate (FDA; Molecular Probes) and 10 μM propidium iodide (PI; Sigma). After 5 min of incubation, both vitally stained cells (by endocytosis of FDA) and cells labeled by loss of membrane integrity (and DNA binding with PI) were counted under fluorescence (450 nm excitation) and compared to total prestained cell counts. Fluorescence photomicrographs were obtained on a Nikon Optiphot microscope. LDH release, a biochemical marker of cell lysis, was assayed on cells grown for 2 days in serum-free medium (28).

Protein Purification and Assay Techniques. Human immunoglobulins were purified from patient sera or plasma as described (6). Immunoglobulins of 90% or greater purity and total protein concentrations of 10–40 mg/ml were produced by these methods. Protein concentration was measured against bovine serum albumin standards with bicinchoninic acid used as the detection reagent (Pierce).

To determine that IgG itself produced cytotoxicity, some IgG fractions were heated to 56°C for 60 min before assay (to determine heat stability), while other 500-μl aliquots of IgG were heated to 96°C for 30 min or treated with 1 mg of trypsin per ml for 1 hr at 37°C (to verify the protein nature of the cytotoxic factor). IgG cytotoxic activity was also tested after mixing 5-mg IgG samples for 1 hr at 4°C with 0.5 ml of protein A coupled to beaded agarose (Pierce) or goat Fc-specific anti-human IgG (Tago) linked to immobilized protein A (10 mg of IgG per ml of agarose). After precipitation of protein A-coupled IgG by centrifugation, supernatants were tested for residual cytotoxic activity; protein A-bound human immunoglobulin was then eluted, redialyzed against PBS, and likewise tested for cytotoxic effects on VSC 4.1 cells.

VGCC subunits were separated from purified L-type VGCC complex (29) by preparative SDS/PAGE (Prep Cell; Bio-Rad) through a 4.5% resolving gel. Purified VGCC or its subunit constituents were tested for their ability to remove cytotoxic antibodies from ALS IgG samples by mixing the IgG with either VGCC or separated VGCC subunits at a ratio of 0.5 μg per ml of channel protein per mg of ALS IgG overnight at 4°C, in the presence of protease inhibitors [antipain, aprotinin, leupeptin, pepstatin (all at 1 μg/ml), 0.2 mM *o*-phenanthroline, 1 μM benzamidine, 1 μM phenylmethylsulfonyl fluoride, and 10 μM iodoacetamide]. Samples were then diluted and tested for toxic effects of digitonin-containing VGCC fractions and for alterations in ALS IgG-mediated toxicity. Potential direct effects of protease inhibitors and digitonin on IgG fraction stability or cell survival were assayed at concentrations identical to those present in VGCC samples.

RESULTS

Effects of ALS Patient Immunoglobulins on VSC 4.1 Cell Survival. In cAMP-differentiated VSC 4.1 cells, addition of ALS IgG produced significant cell loss. When quantitated by serial counts of bright-phase cells beginning before ALS IgG addition, cell loss was first observed 6–12 hr after IgG addition, reaching a maximal effect (40–70% loss) after 48–60 hr (Fig. 1*a*). Prior to death, somata of many cells decreased in size, in part from membrane blebbing and cell process autoamputation. Delayed loss of differentiated VSC 4.1 cells was also dependent on the concentration of added ALS IgG (Fig. 1*b*). While IgG prepared from sera of different patients provided different levels of cytotoxicity, all tested ALS IgGs were maximally toxic at 0.1–0.2 mg of IgG protein per ml of growth medium.

At saturating concentrations of ALS IgG, some remaining cells appeared identical to undifferentiated VSC 4.1 cells and continued to divide. Interestingly, thymidine uptake experiments documented a 50% increase in division rate of such cells after exposure to ALS IgG (R.G.S., M.E.A., and S.H.A., unpublished results). Observed magnitude of total cell loss thus apparently reflected a combination of differentiated cell death and continued mitosis of morphologically undifferentiated clones, in which cell death predominated for the first 2 days. Although these data reflect cell population heterogeneity in the VSC 4.1 hybrid, consistent conditions for cell growth and differentiation produced constant percentage cell loss after saturating ALS IgG treatment. When ALS IgG was added to undifferentiated VSC 4.1 cells, only

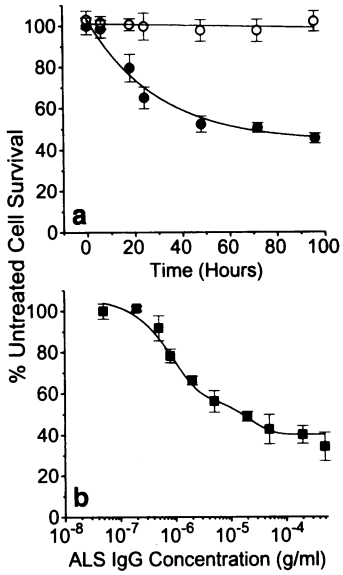

FIG. 1. Cytotoxicity of ALS IgG and IgG obtained from patients with other neurologic diagnoses. (*a*) cAMP differentiated VSC 4.1 cells were maintained without IgG addition or exposed to 0.5 mg of protein per ml of pooled ALS IgG (from four ALS patients; ●) or 0.5 mg of protein per ml of pooled disease control IgG (from four patients with diagnoses of autoimmune polyneuropathy, Guillain–Barré syndrome, myasthenia gravis, or multiple sclerosis; ○). Changes in cell number are depicted as a percentage of untreated culture cell number at each time point. Data fit a single logarithmic decay function (for ALS IgG-dependent cell loss) or line (for disease control IgG-treated cell number). (*b*) ALS IgG-dependent cell loss, tested at IgG concentrations ranging from 0.05 μg/ml to 0.5 mg/ml. Cell counts were obtained as in *a* after addition of single ALS IgG (■). Results at 48 hr are depicted as a percentage of untreated control culture cell number. Data fit a double exponential function by least-squares analysis. In *a* and *b*, each point represents mean cell number surviving in triplicate cultures (cell number in each of 7–10 1-mm² fields = the value for a single culture) in three experiments (total of nine cultures) ± SD.

a small (10–15%) reduction in cell number was noted, and this small change was largely obscured by continued cell division. No toxic effect of ALS IgG was noted when assayed on undifferentiated or cAMP-treated N18TG2 parent cells or when tested with similarly treated MES 23.5 hybrid cells prepared from embryonic mesencephalon neurons and the N18TG2 line (26).

Although ALS IgG usually was maintained in culture medium throughout cell survival assays, prolonged incubation was not required for the delayed cytolysis. Pulse addition of 0.2 mg of ALS IgG per ml was as effective as continuous immunoglobulin presence in producing cytolysis. Exposure of differentiated VSC 4.1 cells to ALS IgG for as little as 10 min produced a 28% ± 4.5% reduction in cell number measured after 2 days, while incubation of cells for 30 min provided maximal (51% ± 2.2%) cytolytic activity.

Using vital dyes to assess viability of counted cells, ALS IgG-mediated cell loss was found to be more consistent with cell death rather than simple cell substratum detachment (Figs. 2 and 3). ALS IgG addition produced a time-dependent loss in cells labeled by energy-dependent endocytosis of FDA, concomitant with increased numbers of dead and dying cells containing the DNA label PI. As assayed in the same cultures, a close correspondence was observed between cell loss measured with whole cell counts and loss of cell viability assayed with vital dyes.

When cells were grown for 18 hr in serum-free medium containing ALS IgG, mean LDH activity retained in intact cells closely paralleled residual mean cell survival, as determined by direct counts of bright-phase VSC 4.1 cells. Both assays documented equivalent ALS IgG concentration-dependent reductions in cell survival. At maximally toxic IgG concentrations (0.1–0.2 mg of ALS IgG protein per ml), both assays also provided equivalent maximal percentage reductions in cell number (27.8% ± 3.1%) and in retained LDH activity (26.6% ± 1.3%). Likewise, release of LDH into the growth medium (representing cell lysis) paralleled increases in cell loss, with a maximal observed increase in LDH release of 28.2% ± 6.2%.

Specificity of ALS IgG-Mediated Toxicity. The loss of VSC 4.1 cells was relatively specific for ALS IgG (Fig. 3), and at saturating toxic concentrations all ALS IgG produced similar magnitudes of cell loss. However, cell survival and cell division rate were not altered after addition of IgG purified from normal individuals or from most patients with other neurologic diseases, even after exposure for as much as 5 days. Exceptions to this rule were limited to IgG from five of six patients with Lambert–Eaton myasthenic syndrome (LEMS IgG) whose antisera were previously shown to contain anti-VGCC antibodies (6). Again, in these experiments, identical magnitudes of cytotoxicity were observed when performing whole cell counts (Fig. 3*a*) and when assaying cell viability (Fig. 3*b*).

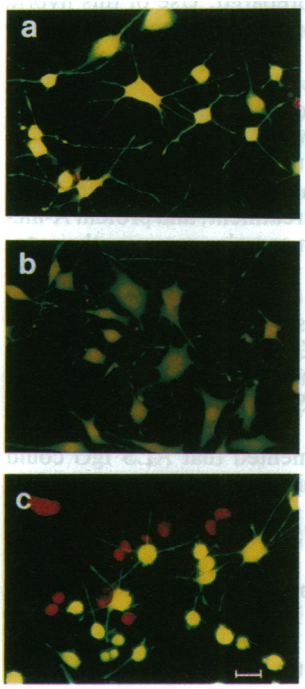

FIG. 2. Photomicrographs depict fluorescent labeling of VSC 4.1 cells by energy-dependent endocytosis of FDA (green) and by loss of membrane integrity and DNA staining with PI (red) after 2-day exposure to 0.5 mg of protein per ml of IgG from a healthy individual (*a*), a patient with Guillain–Barré syndrome (*b*), and a patient with ALS (*c*). Note increased numbers of rhodamine-stained cells, with loss of plasma and nuclear membrane integrity. (Bar = 50 μm.)

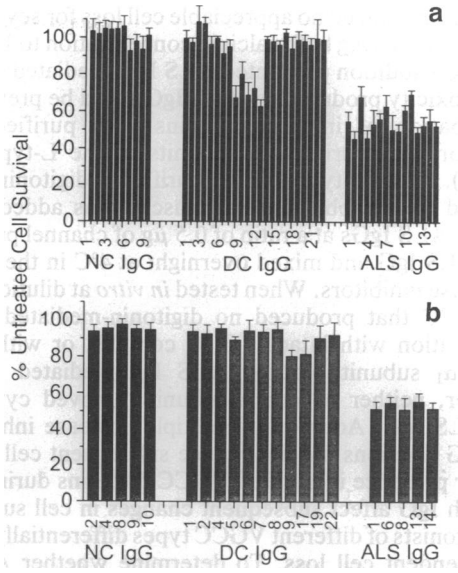

FIG. 3. (*a*) Cell survival assay using IgG prepared from 15 patients with ALS (ALS IgG), 10 age- and sex-matched healthy individuals (NC IgG), and 23 age- and sex-matched patients with other neurologic diseases (DC IgG). IgG-related cell survival is depicted as a percentage of untreated control culture cell number after 2-day IgG treatment. Bars represent cell survival data for individual patient IgG, tested at 0.5 mg of protein per ml in triplicate cultures in each of four experiments ± SD. (*b*) Fluorescent dye assay of cell viability using IgG prepared from randomly selected patients from *a*. Two days after addition of 0.5 mg of protein per ml of IgG, FDA and PI were added to cells, and viable cell number was determined by the ratio (counts of fluorescent FDA-labeled cells)/(FDA-labeled cells and PI-labeled cells). Bars represent viable cell number for individual patient IgG, expressed as a percentage of untreated culture values and tested in duplicate coverslips for each of two experiments ± SD. Viable cell counts were identical to bright-phase cell counts, as measured in the same cultures (data not shown). Neurologic disease IgG (DC IgG): autoimmune polyneuropathy, DC 1 and 2; multiple sclerosis, DC 3; Guillain–Barré syndrome, DC 4–7; LEMS, DC 8–13; Parkinson disease, DC 14 and 15; myasthenia gravis, DC 16–19; Alzheimer disease, DC 20–23.

Mechanism of ALS IgG Effect on VSC 4.1 Cytolysis. ALS IgG-mediated effects were independent of complement, with cytolysis observed in serum-free Sato's medium, in medium containing 56°C heat-inactivated newborn calf serum, and after preheating ALS IgG fractions to 56°C for 60 min. However, ALS IgG-mediated cell cytotoxicity was lost after boiling IgG for 30 min or treating IgG with 1 mg of trypsin per ml for 1 hr at 37°C and was reduced by >90% with prior quantitative removal of IgG either by direct precipitation with protein A or with anti-human IgG coupled to protein A. Cytotoxicity was retained in the protein A-bound fractions, as determined by testing activity of those fractions after acid elution and dialysis.

ALS IgG-mediated cell loss was also calcium dependent. Reduction of extracellular calcium concentration with EGTA prevented IgG-induced cell death. Addition of EGTA sufficient to buffer extracellular calcium concentrations to 10 μM to 100 nM (as verified by Fura-2 fluorescence against known

standards) produced no appreciable cell loss for several days. However, buffering bath calcium concentration to 10 μM 6 hr before IgG addition prevented ALS IgG-mediated cytolysis.

Cytotoxicity produced by ALS IgG could be prevented by preincubation of immunoglobulins with purified L-type VGCC or with purified α_1 subunits of the L-type VGCC (Table 1). Intact L-type VGCC, purified in digitonin micelles extracted from rabbit skeletal muscle, was added to three different ALS IgGs at a ratio of 0.5 μg of channel protein per mg of ALS IgG and mixed overnight at 4°C in the presence of protease inhibitors. When tested *in vitro* at dilutions of IgG and VGCC that produced no digitonin-mediated toxicity, preincubation with intact VGCC complex or with purified VGCC α_1 subunit blocked ALS IgG-mediated cell loss. However, neither α_2 nor β subunit removed cytotoxicity from ALS IgG. Addition of multiple protease inhibitors to ALS IgG fractions did not reduce subsequent cell loss, nor did their presence in purified VGCC fractions during admixture with IgG affect subsequent changes in cell survival.

Antagonists of different VGCC types differentially affected IgG-dependent cell loss. To determine whether ALS IgG-mediated cytolysis might involve calcium entry through VGCCs, 0.1–10 μM nifedipine (a blocker of dihydropyridine-sensitive L-type VGCCs), 0.1–20 μM Bay K8644 (an activator of L-type VGCCs), 2 pM to 500 nM ω-conotoxin (a potent inhibitor of N-type VGCC function), and 0.5–100 nM Aga-IVa toxin (a P-type VGCC antagonist) were separately tested on cAMP-treated VSC 4.1 cells in both the absence and presence of ALS IgG (Fig. 4). At concentrations that did not directly affect cell survival, ω-conotoxin added concomitantly with six different ALS IgGs completely blocked ALS IgG-induced VSC 4.1 cell death. This effect of ω-conotoxin was itself concentration dependent, saturating between 200 pM and 2 nM. Likewise, Aga-IVa addition to cells before ALS IgG addition greatly reduced subsequent cell death in a toxin concentration-dependent fashion that saturated at 10–30 nM. Neither Bay K8644 nor nifedipine altered ALS IgG-induced cytotoxicity.

Since similar magnitudes of differentiated VSC 4.1 cell loss were observed after addition of ALS IgG, 1 μM AMPA, 2 μM quisqualate, or 10 μM kainate (R.G.S., unpublished data), ALS IgG was also tested on cells pretreated with 0.1–10 μM CNQX (an AMPA/kainate receptor antagonist). CNQX (10 μM) did not appear to alter ALS IgG-mediated cytotoxicity, even though it blocked AMPA-induced cell death and was not itself toxic.

Effects of ω-conotoxin on LEMS IgG-induced cell death differed from those observed with ALS IgG. At concentrations that prevented ALS IgG-mediated cytolysis, ω-conotoxin increased the rate of cell loss observed with concomitant administration of three different toxic LEMS IgGs; 0.2 mg of protein per ml of ALS IgG produced a 45% ± 5.2% cell loss (compared to untreated controls) after 2 days, while 0.2 mg of protein per ml of LEMS IgG induced a 34% ± 5.8% cell loss in the same time interval. However, while ALS IgG-mediated cytolysis was completely blocked by simultaneous addition of 200 pM ω-conotoxin, addition of toxin with LEMS IgG increased subsequent cell loss to 43% ± 4.6% of untreated cell counts. In these experiments, values represent the mean cell survival ± SD for three experiments (mean of 27 culture well cell counts). Thus, antibodies produced by patients with LEMS and ALS are both cytotoxic to VSC 4.1 cells, but ALS IgG toxicity is blocked by ω-conotoxin while effects of LEMS IgG are not reduced by ω-conotoxin.

Table 1. Effect of ALS IgG premixture with VGCC or VGCC subunits on subsequent cell survival

	VGCC component addition* (*n*)	ALS IgG + VGCC component addition† (*n*)
No VGCC	100.0 ± 6.9 (9)	58.2 ± 5.2 (6)
VGCC complex	93.4 ± 2.8 (6)	97.5 ± 4.3 (6)
VGCC α_1 subunit	94.9 ± 1.6 (6)	94.1 ± 2.1 (6)
VGCC α_2 subunit	95.8 ± 1.5 (6)	57.1 ± 6.1 (6)
VGCC β subunit	98.0 ± 3.9 (6)	55.1 ± 4.7 (6)

n, Number of cultures assayed in three experiments.
*Cell survival: values represent mean cell counts ± SD, expressed as a percentage of untreated culture cell numbers after 3 days treatment.
†See text for methodology and IgG/VGCC concentrations tested.

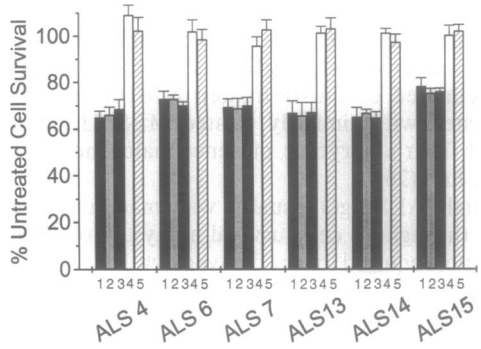

FIG. 4. Effects of VGCC modulators on ALS IgG-mediated cytotoxicity: 0.2 mg of protein per ml of ALS IgG was added to VSC 4.1 cells after 15-min treatment at room temperature without VGCC modulators (*1*), with 10 μM nifedipine (*2*), with 20 μM Bay K8644 (*3*), with 200 pM ω-conotoxin (*4*), or with 20 nM Aga-IVa toxin (*5*). Cell survival after 2-day exposure to six different ALS IgGs (ALS 4, 6, 7, 13, 14, and 15) tested in the presence or absence of VGCC modulators is shown. At indicated concentrations, VGCC modulators themselves did not affect cell survival or proliferation. Results represent means of four experiments, assaying triplicate culture wells per experiment, ± SD.

DISCUSSION

The VSC 4.1 motoneuron–neuroblastoma hybrid line offers a whole cell model system with which to assess vulnerability resulting from addition of ALS IgG. Cell loss from IgG is selective, with selectivity apparently conferred by both the type and differentiation state of the cells and by the type of disease from which IgGs were prepared. Use of this hybrid motoneuron model may overcome reported controversy in documenting specific effects of ALS serum components on embryonic spinal cord neurons (30–32), possibly resulting from primary culture cell heterotypy and from difficulty in differentiation to a stable mature *in vitro* phenotype.

Differentiated VSC 4.1 cells treated with ALS IgG fractions die within 24–48 hr. IgG appears to initiate cell injury directly, since boiling, protease treatment, and protein A-mediated IgG preextraction of such samples removes the cytotoxic factor, while complement inactivation does not affect ALS IgG-dependent cytolysis. Cytotoxicity is also dependent on extracellular calcium, since reducing extracellular calcium concentration with EGTA protects against ALS IgG-induced cell death. Together, these data suggest that ALS IgG may directly initiate VSC 4.1 cell injury by enhancing calcium entry into cells.

While previous studies documented that ALS IgG could inhibit calcium flux through skeletal muscle L-type VGCCs (7–9), the present experiments suggest that ALS IgG may enhance calcium current in VSC 4.1 cells by activating neuronal N- or P-type VGCCs. Stimulatory effects of ALS IgG have in fact been observed on neuronal calcium currents in VSC 4.1 cells (33) and on P-type calcium currents in Purkinje cells and lipid bilayers (34). The ability of ω-conotoxin and Aga-IVa to block cytotoxic effects of ALS IgG

suggests that ALS IgG-mediated cytotoxicity is dependent on entry of calcium selectively through neuronal calcium channels or through different calcium ionophores whose function is modulated by N- or P-type VGCC activity. The inability of dihydropyridines to influence VSC 4.1 cell death suggests that L-type VGCCs are not functionally involved in this process.

Preincubation of ALS IgG with whole purified L-type VGCC or with purified L-type VGCC α_1 subunit (but not with α_2 or β subunit) selectively removes cytotoxic effects. These data are consistent with the observation that ALS IgG selectively recognizes the VGCC α_1 subunit (35). However, although VSC 4.1 cells possess dihydropyridine-sensitive L-type VGCC binding sites, IgG-mediated cytotoxicity is dihydropyridine insensitive and apparently not mediated by L-type VGCCs. The observation that removal of cytotoxic IgG by preincubation with L-type VGCC mimics the anticytolytic effects of inhibitors of N- or P-type but not L-type VGCCs instead suggests that ALS IgG-mediated cell loss may depend on immunoglobulin binding to epitopes common to all these VGCCs rather than to immunoglobulin specificity for a single VGCC subtype. Extracellularly directed regions of primary sequence homology exist between L-, N-, and P-type VGCC α_1 subunits, although definitive proof for multiple-type VGCC cross-reactive antibodies in ALS patients is still lacking.

From this discussion, several potential explanations may be proposed to explain why N18TG2 neuroblastoma, undifferentiated VSC 4.1 cells, and differentiated MES 23.5 cells are resistant to the cytotoxic effects of ALS IgG, while cAMP-treated VSC 4.1 cells are sensitive to ALS IgG addition. Quantitating differences in the number of L-type, N-type, and P-type channels between treated and untreated cells may not completely explain why only differentiated VSC 4.1 cells die. Better hypotheses for selective vulnerability include differences in the types of expressed VGCCs, differences in specific functions performed by each class of VGCC, differences in second messenger-mediated modulation of VGCC function after cAMP treatment, or differences in calcium-buffering capacity, possibly related to altered levels of calcium binding protein (36).

Cytotoxic effects initiated by IgG were relatively specific for ALS, since only LEMS IgG gave a comparable effect. Several Guillain–Barré syndrome IgGs previously found to bind L-type VGCCs in ELISA (6) were not toxic to VSC 4.1 cells, suggesting that they might interact at a different, noncytotoxic epitope. While LEMS IgGs also contain anti-VGCC antibodies (37–39), and all tested LEMS IgGs were previously found to bind L-type VGCCs in ELISA, such antibodies may induce antigenic modulation of neuronal VGCCs and reduce voltage-activated calcium flux into motoneurons (37, 38). That ω-conotoxin did not block but instead accentuated cytotoxicity of LEMS IgG in VSC 4.1 cells is in accord with these suggested opposite effects of LEMS and ALS IgGs on motoneuron VGCC function and is consistent with previous passive transfer studies that documented opposite effects of these IgGs on VGCC-mediated acetylcholine release at neuromuscular junctions (18, 40). Thus, while ALS IgG and LEMS IgG are both toxic to VSC 4.1 cells, they appear to use different mechanisms to exert their effects.

Recently, IgM from patients with type I diabetes have been demonstrated to increase activity of L-type calcium channels in pancreatic β cells (41). Our demonstration that ALS IgG is selectively toxic for this differentiated motoneuron cell line, and that such cytotoxicity is dependent on calcium and mediated by neuronal type (N-type and P-type) voltage-gated calcium channels, suggests that autoimmune mechanisms may contribute to the destruction of motoneurons in ALS, just as immunoglobulin-mediated increases in calcium influx may contribute to the destruction of β cells in insulin-dependent diabetes mellitus.

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