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Expression of calbindin-D_{28K} in motoneuron hybrid cells after retroviral infection with calbindin-D_{28K} cDNA prevents amyotrophic lateral sclerosis IgG-mediated cytotoxicity

(calcium-binding proteins/motoneuron degeneration)

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ABSTRACT **Calbindin-D_{28K} and/or parvalbumin appear to influence the selective vulnerability of motoneurons in amyotrophic lateral sclerosis (ALS). Their immunoreactivity is undetectable in motoneurons readily damaged in human ALS, and in differentiated motoneuron hybrid cells [ventral spinal cord (VSC 4.1 cells)] that undergo calcium-dependent apoptotic cell death in the presence of ALS immunoglobulins. To provide additional evidence for the role of calcium-binding proteins in motoneuron vulnerability, VSC 4.1 cells were infected with a retrovirus carrying calbindin-D_{28K} cDNA under the control of the promoter of the phosphoglycerate kinase gene. Differentiated calbindin-D_{28K} cDNA-infected cells expressed high calbindin-D_{28K} and demonstrated increased resistance to ALS IgG-mediated toxicity. Treatment with calbindin-D_{28K} antisense oligodeoxynucleotides, which significantly decreased calbindin-D_{28K} expression, rendered these cells vulnerable again to ALS IgG toxicity.**

Despite extensive investigations of superoxide dismutase mutations in familial amyotrophic lateral sclerosis (ALS) and excitotoxicity and autoimmunity in sporadic ALS, our understanding of the factors dictating selective vulnerability of motoneurons are incompletely understood (1–5). Calbindin-D_{28K} and/or parvalbumin have been implicated in ALS pathogenesis since immunoreactivity for these calcium binding proteins is absent in neurons early and severely affected in ALS such as ventral horn motoneurons and is present in neurons late or infrequently affected such as oculomotor neurons or Onuf's neurons (6, 7). In addition, motoneuron hybrid cells that are selectively killed by ALS IgG have little or absent immunoreactivity for calbindin-D_{28K} and parvalbumin whereas cells not affected by ALS IgG, including undifferentiated motoneuron hybrid cells and the parent neuroblastoma N18TG2 cells, have high levels of calbindin-D_{28K} and parvalbumin (7).

We report herein that retroviral infection of motoneuron hybrid cells with calbindin-D_{28K} cDNA induces increased calbindin-D_{28K} expression that is maintained after cAMP differentiation and prevents ALS IgG-induced toxicity. Conversely, inhibition of calbindin-D_{28K} expression by treatment of calbindin-D_{28K}-infected ventral spinal cord (VSC) 4.1 cells (I-VSC 4.1 cells) with calbindin-D_{28K} antisense oligodeoxynucleotides restores vulnerability to ALS IgG-mediated toxicity.

MATERIALS AND METHODS

Construction of pStMCS-PCalb for Retrovirus Packaging. The calbindin-D_{28K} cDNA was cloned from rat brain as follows: total RNA was extracted from rat brain by the guanidinium/cesium chloride method (8). Poly(A)$^+$ RNA was purified by oligo(dT) cellulose (Collaborative Research, Bedford, MA) affinity chromatography. The entire coding sequence (786 bp) of calbindin-D_{28K} was synthesized from the poly(A)$^+$ RNA by reverse transcriptase (GIBCO/BRL)–polymerase chain reaction (PCR, Perkin–Elmer/Cetus) and cloned into the *Bam*HI site of plasmid pGEM-3Z (Promega). The sequences of the oligomers for PCR are TTCGGATCC-ATGGCAGAATCCCACCTGCA for the 5′ forward primer and AAAGGATCCTAGTTGTCCCCAGCAGAGAGAAT for the 3′ reverse primer (the oligomers were synthesized at the Department of Cell Biology, Baylor College of Medicine). A clone containing the correct complete calbindin-D_{28K} sequence was identified by dideoxynucleotide sequencing (9).

The calbindin-D_{28K} cDNA was obtained from the clone pGEM-calbindin by *Bam*HI digestion and cloned into the *Sma*I site of pPGKbpA (derived from pPGKneo) after filling-in of ends by treatment with the Klenow fragment of DNA polymerase I (Promega). As shown in Fig. 1, this enabled insertion of the calbindin-D_{28K} cDNA downstream of the PGK promoter/enhancer sequence and upstream of a polyadenylylation signal of the growth hormone gene. Orientation of the cDNA was determined by digestion with *Eco*RI. The resulting construct was digested with *Xho*I, treated with Klenow polymerase, and subsequently, digested with *Hin*dIII. The resulting mixture of fragments were ligated into *Stu*I- and *Hin*dIII-digested vector pStMCS. The plasmid pStMCS (kindly provided by John Belmont, Baylor College of Medicine, Houston, TX) is a retroviral vector, containing Moloney murine leukemia virus long terminal repeats. The orientation of the calbindin expression cassette is opposite to that of the long terminal repeat sequences in the final pStMCS-PGK-calbindin (pStMCS-PCalb) plasmid (Fig. 1).

Production of Recombinant Retrovirus and Infection of VSC 4.1 Cells with Calbindin-D_{28K}-Expressing Retrovirus. The plasmid pPGKneo and the ecotropic virus packing cell line GP+E86 were provided by John Belmont. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum in 10% CO_2/90% air.

Twenty micrograms of pStMCS-PCalb and 1 μg of pPGKneo were cotransfected into 3.5×10^6 ecotropic packing GP + E86 cells by electroporation (Invitrogen electroporator II) at $\infty\Omega$, 1000 μF, and 280 V. Transfection efficiency varied from 7 to more than 200 neomycin-resistant clones per electroporation. Several hundred neomycin (GIBCO/BRL, 4 μg/ml)-resistant clones were obtained. The recombinant virus pro-

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Abbreviations: ALS, amyotrophic lateral sclerosis; ODN, oligodeoxynucleotides; PGK, phosphoglycerate kinase; VSC, ventral spinal cord.

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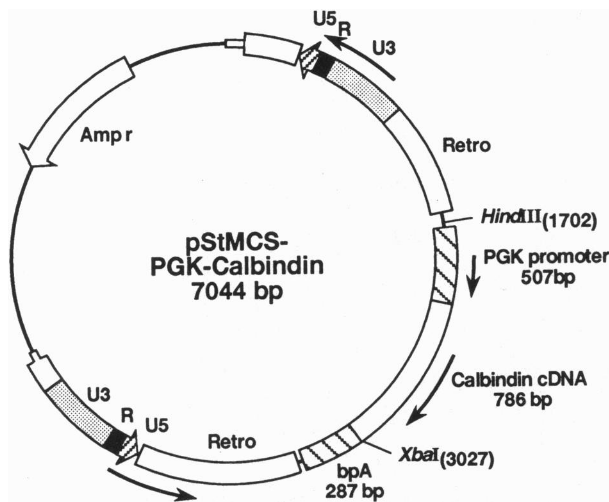

FIG. 1. Construction of retroviral vector for expression of the calbindin-D_{28K} gene under the regulation of the promoter of the phosphoglycerate kinase (PGK) gene.

duced by transient expression of the infected GP + E86 cells was harvested after 48 h from conditioned medium by filtration through 0.45-μm (pore size) Millipore filters. As this virus does not carry a selectable marker, we amplified the diluted supernatant by ping-pong infection in a mixture of 50% GP + envAm12 and 50% GP + E86 cells and titrated the virus by scoring the wells positive to retroviral sequences by PCR. The VSC 4.1 cells were infected by exposure to virus with a titer of 10^6 virus particles per ml for 4 h in the presence of Polybrene at 4 μg/ml (to neutralize cell surface charge). Among the multiple PGK-calbindin retrovirus-infected cell lines, we have chosen the line named I-VSC 4.1, since after differentiation with dibutyryl cAMP and aphidocolin, the level of calbindin immunoreactivity did not decrease as it does in noninfected VSC 4.1 cells. We have used the I-VSC 4.1 cells in all subsequent experiments.

Evidence of Calbindin-D_{28K} cDNA and mRNA in VSC 4.1 and I-VSC 4.1 Cells. *Southern blot analysis.* Genomic DNA from VSC 4.1 and I-VSC 4.1 cells were extracted by phenol/chloroform, followed by RNase treatment. For Southern blot analysis, gDNA was digested overnight at 37°C with *Hin*dIII and *Xba*I and separated on an 1% agarose gel. The DNA was denatured and transferred to nitrocellulose membrane. After fixing and blocking, the filter was probed with ^{32}P calbindin-D_{28K} cDNA, labeled by nick-translation, overnight at 65°C in 6× SSC/1× Denhardt's solution. Washes were carried out at room temperature for four 20-min periods in 2× SSC and for 30 min at 65°C in 0.5× SSC. Autoradiography was performed overnight at −70°C.

Northern blot analysis. The mRNA of the VSC 4.1 and I-VSC 4.1 cells was isolated as mentioned before, but with one more cycle of oligo(dT) affinity chromatography. For Northern blot analysis, 15 μg of mRNA was electrophoresed on a 1.2% formaldehyde agarose gel and transferred to a nylon filter. The filter was probed with ^{32}P calbindin-D_{28K} cDNA at 42°C in 50% formamide/6× SSPE. Filters were washed for four 30-min periods at room temperature in 2× SSC and for one 30-min period in 0.5× SSC at 60°C. The autoradiography exposure time was 2 days at −70°C.

In situ *hybridization.* The presence of calbindin-D_{28K} mRNA in the motoneuron hybrid cells with or without introduction of the calbindin-D_{28K} gene was also documented by nonradioactive *in situ* hybridization (10, 11) using fluorescein-labeled oligodeoxynucleotide (ODN) probes (Genosys, The Woodlands, TX) and the oligocolor kit from Amersham. The ODN probes used in our assay consisted of 33 nt complementary to the coding region for amino acids 31–41 of the calbindin-D_{28K} gene sequence (12). The 5′-3′ sequence of the antisense ODN used was CTGGATCAAGTTCTGCAGCTCCTTTCCT-TCCAG. The hybridization was performed by the instructions in the Amersham kit. The *in situ* buffer provided by Amersham contained 50% formamide, 2× SSC, 1× Denhardt's solution, herring testes DNA (300 μg/ml), and an enhancement compound. Fifty microliters of hybridization solution with a final probe concentration of 50 ng/ml was applied on each coverslip and the incubation was performed for 5 h at 37°C. The slides were washed in 1× SSC/0.1% SDS at room temperature and then in 0.2× SSC/0.1% SDS at 37°C. Signal was detected with a biotinylated anti-fluorescein antibody (Molecular Probes) followed by avidin-biotin complex (Vector Laboratories) and 3,3′-diaminobenzidine (Sigma).

Control experiments were carried out to assure the specificity of the probes and the appropriateness of the hybridization conditions and detection methods as follows: To control for the specificity of the effect of calbindin-D_{28K} expression, we investigated the levels of mRNA for a cytoskeletal protein (heavy-chain neurofilament) (13) and for another calcium-binding protein normally expressed in VSC 4.1 cells (parvalbumin) (14). Experimental procedures performed with fluorescein-labeled sense ODN, complementary to the antisense probes for calbindin-D_{28K}, parvalbumin, and neurofilament, respectively, or experiments without the hybridization buffer, or omitting the antibody against fluorescein resulted in complete absence of any signal.

Cell Culture Conditions and Assessment of the Effects Produced by Calbindin-D_{28K} Gene Transfection. General cell growth, cAMP, and aphidocolin differentiation conditions as well as the preparation for survival/cytotoxicity assays and immunohistochemical experiments were performed as described in our previous studies (7, 15, 16).

To reverse the effect of calbindin-D_{28K} gene transfection, the I-VSC 4.1 cells were treated after differentiation with 20 μM calbindin-D_{28K} antisense oligodeoxynucleotides (Genosys) for 24 h before further treatments or procedures. The antisense ODN sequence used was 5′-CAGGTGTTCTGC-CAT-3′, which starts from the putative translation initiation codon according to the published calbindin-D_{28K} gene sequence (12). In all experiments, sister cultures were treated with a complementary calbindin-D_{28K} sense ODN probe at 20 μM. Neither the antisense probe nor the sense probe demonstrated any toxic effects on the survival of motoneuron hybrid cells. Furthermore, cultures treated with calbindin-D_{28K} sense ODN did not show changes in immunoreactivity for calbindin-D_{28K}. These results are consistent with other reports using ODN to modulate specific protein expression in neuronal-type tissue culture experiments (17–19).

The quantitation of calbindin-D_{28K} in the motoneuron hybrid cells was determined by a sensitive immuno-sandwich assay as described by Zhu *et al.* (20). Briefly, the monoclonal anti-calbindin-D_{28K} antibody (Sigma) was adsorbed onto silicone tube pieces; the same antibody was chemically crosslinked to β-galactosidase used as a reporter enzyme (21). The bivalent antigen was sandwiched between the antibody adsorbed to the silicone piece and the antibody crosslinked to β-galactosidase (22). The recorded enzyme activity measures the level of antigen after deducting the background activity obtained in the absence of antigen. The optical density values obtained without cell extract or purified calbindin-D_{28K} were taken as background values in each experiment and were deducted from the values obtained for our specific experimental probes.

Electrophysiological recordings were made on the stage of a World Precision Instruments (WPI) inverted microscope. The extracellular fluid contained 40 mM $BaCl_2$, 107.5 mM tetraethylammonium chloride, and 10 mM Hepes. Tetrodotoxin (1 mM) was added to inhibit sodium currents. Patch recordings were performed with low-resistance (1–2 MW)

pipettes pulled from borosilicate glass (WPI), using a Flaming/Brown puller (P.80/PC, Sutter). Whole-cell patch recordings were made from the cells by using patch electrodes filled with a solution containing 120 mM cesium methanesulfonate, 4.5 mM $MgCl_2$, 9 mM glucose, 9 mM EGTA, 9 mM Hepes, and 4.5 mM ATP. Patch electrodes and extracellular solutions were adjusted to pH 7.3. Signals were recorded using a patch-clamp amplifier (PC-501A, Warner) and a laboratory computer (486/66) equipped with a Labmaster A/D converter. Voltage pulses of 50- or 500-ms duration were applied from a holding potential (V_h) of −70 mV, at frequencies of 0.1–0.3 s^{-1}. Seal resistances and capacitative transients were monitored during experiments by applying small (5 mV) voltage pulses superimposed on the command potential. Current–voltage (*I–V*) relationships were obtained by plotting the peak current evoked by a voltage pulse against the pulse potential. Digital leak subtraction was carried out prior to current–voltage analysis by scaling and subtraction of responses to small 5–10 mV hyperpolarizing pulses.

For toxicity studies uninfected and infected differentiated motoneuron hybrid cells, in triplicate wells for each immunoglobulin used in each experiment, were treated within 24 h after plating with IgG at 0.2–0.5 mg/ml purified from patients with ALS, as reported (23). The six ALS IgGs used in this study were chosen at random from our stock of immunoglobulins. The mean age of the patients was 63 ± 11 years, and all patients fulfilled clinical and laboratory criteria for ALS. Cell survival was assessed after incubation for 48 h with ALS IgG by counting five contiguous 1-mm^2 fields predetermined and marked after plating at day zero. This direct counting assay has been well correlated with vital staining using fluorescein diacetate and propidium iodide and with the lactate dehydrogenase release assay (15).

RESULTS

Expression of Calbindin-D_{28K} cDNA. VSC 4.1 cells were infected with a retroviral vector with calbindin-D_{28K} cDNA under the control of a PGK promoter (Fig. 1); the clone I-VSC 4.1 was selected for further analysis. Southern blot analysis of the VSC 4.1 and I-VSC 4.1 cells showed that the average copy number of genomic DNA from PGK–calbindin genes integrated into the original VSC 4.1 genomic DNA is three copies per cell. Northern blot analysis of VSC 4.1 cell mRNA demonstrated a single positive band of 1.8 kb that decreased significantly in differentiated VSC 4.1 cells, whereas the Northern blot analysis of I-VSC 4.1 cells yielded two bands, a 1.8-kb minor band and a 1.4-kb major band, that decreased only slightly in differentiated I-VSC 4.1 cells (Fig. 2).

Nonradioactive *in situ* hybridization for calbindin-D_{28K} mRNA demonstrated significantly higher levels in I-VSC 4.1 cells than in noninfected cells (Fig. 3 *A* and *C*). In contrast to differentiated noninfected VSC 4.1 cells, calbindin-D_{28K} mRNA expression in I-VSC 4.1 cells remained high after differentiation with cAMP (Fig. 3 *B* and *D*). In control experiments, the level of heavy-chain neurofilament protein mRNA as well as parvalbumin mRNA remained unchanged in I-VSC 4.1 cells. Similarly, the increase in neurofilament expression with cAMP differentiation reported in VSC 4.1 cells (15, 24) remained present also at the mRNA level in I-VSC 4.1 cells.

Effects of Calbindin-D_{28K} Gene Expression in I-VSC 4.1 Cells. *Cell Growth Rate.* The I-VSC 4.1 cell lines demonstrated no difference in the rate of division when compared with the noninfected VSC 4.1 cells. In triplicate experiments with triplicate wells, we found that the fold increase in cell number per mm^2 after 4 days in culture is 4.84 ± 0.7 for the undifferentiated I-VSC 4.1 cells compared to 4.83 ± 1.2 for the undifferentiated VSC 4.1 cells.

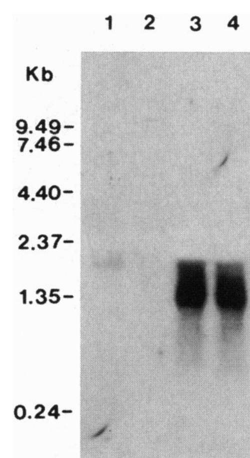

FIG. 2. Evidence for the calbindin gene transfection in motoneuron hybrid cells. Northern blot of the mRNA extracted from undifferentiated VSC 4.1 cells (lane 1), differentiated VSC 4.1 cells (lane 2), undifferentiated I-VSC cells (lane 3), and differentiated I-VSC cells (lane 4).

Immunohistochemical Levels of Calbindin-D_{28K}, Parvalbumin, Calmodulin, and Neurofilament. Retroviral infection of VSC 4.1 cells with calbindin-D_{28K} cDNA resulted in higher levels of calbindin-D_{28K} immunoreactivity in the I-VSC 4.1 cells (Fig. 4). Differentiated I-VSC 4.1 cells showed intense immunostaining for calbindin-D_{28K}, comparable to the levels of undifferentiated I-VSC 4.1 cells and even stronger than the undifferentiated noninfected VSC 4.1 cells, which are resistant to ALS IgG toxicity (7, 15). After treatment with calbindin-D_{28K} antisense ODN, immunoreactivity for calbindin-D_{28K} decreased significantly in differentiated I-VSC 4.1 cells, while calbindin-D_{28K} sense ODN did not alter calbindin-D_{28K} immunoreactivity.

The qualitative assessment of the effects of calbindin-D_{28K} gene expression obtained by *in situ* hybridization, Northern blot analysis, and immunohistochemistry was confirmed by quantitative analysis using an immuno-sandwich assay. The mean calbindin-D_{28K} was 22. 5 ± 3 ng/mg of cell extract protein in undifferentiated VSC 4.1 cells and 60 ± 10 ng/mg of cell extract protein in undifferentiated I-VSC 4.1 cells. In

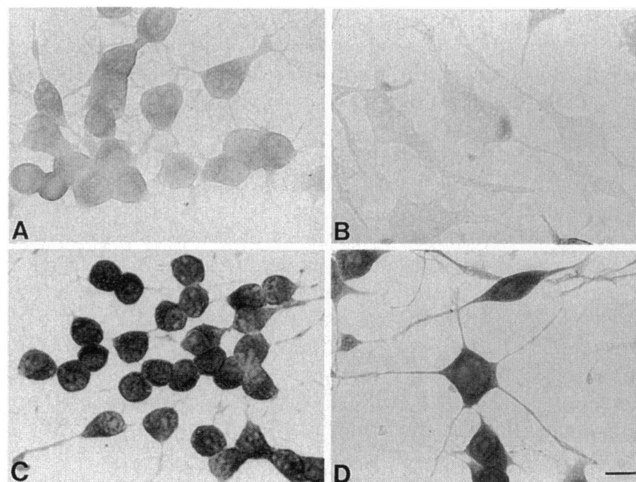

FIG. 3. *In situ* hybridization of calbindin-D_{28K} mRNA in motoneuron hybrid cells: undifferentiated VSC 4.1 cells (*A*), differentiated VSC 4.1 cells (*B*), undifferentiated I-VSC cells (*C*), differentiated I-VSC cells (*D*). Experimental procedures performed with fluorescein-labeled sense ODN, without the hybridization step, or by omitting the antibody against fluorescein resulted in complete absence of any signal. (Bar = 10 μm.)

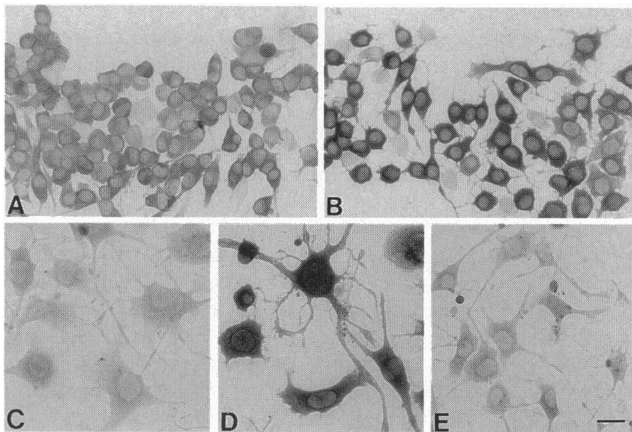

FIG. 4. Immunohistochemical expression of calbindin-D_{28K} in undifferentiated VSC 4.1 cells (*A*), undifferentiated I-VSC cells (*B*), differentiated VSC 4.1 cells (*C*), differentiated I-VSC cells (*D*), and differentiated I-VSC cells treated with calbindin-D_{28K} antisense oligodeoxynucleotide (*E*). Specificity of the monoclonal anti-calbindin-D_{28K} antibody used in these experiments has been described (7, 25, 26). (Bar = 15 μm.)

VSC 4.1 cells, cAMP differentiation induced a 54.3 ± 4% decrease in calbindin-D_{28K} expression (Fig. 5), while retroviral infection resulted in almost three times (283.1 ± 9%) more calbindin-D_{28K} protein expression in infected cells compared to the undifferentiated noninfected VSC 4.1 cells. Furthermore, incubation of differentiated I-VSC 4.1 cells with calbindin-D_{28K} antisense ODN induced a 55.7 ± 11% decrease in calbindin-D_{28K} protein expression compared with the undifferentiated I-VSC 4.1 cells and a 40 ± 5% decrease compared to differentiated I-VSC 4.1 cells. Thus, both immunohistochemical methods and quantitative immunoassays yielded the same pattern of calbindin-D_{28K} protein expression in different cell types.

Immunoreactivity for other cytoplasmic proteins with structural or functional roles in motoneuron cells, such as neurofilaments or parvalbumin, and calmodulin remained unchanged after calbindin-D_{28K} gene transfection (data not shown). Furthermore, the cAMP-inducible levels of choline acetyltransferase activity [evaluated using Fonnum's method

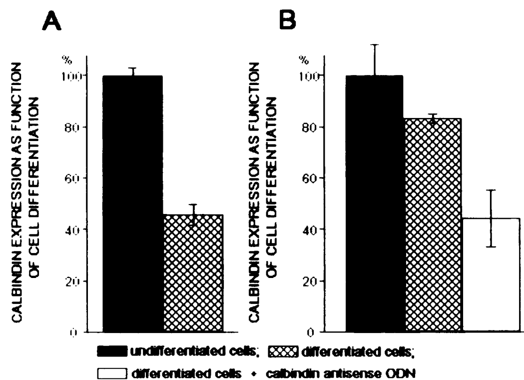

FIG. 5. Quantitative immunoassay for calbindin-D_{28K} expression shows that differentiated VSC 4.1 cells have significantly decreased levels of calbindin-D_{28K} protein (*A*), whereas in calbindin-D_{28K} retrovirus-infected cells, a significant decrease in calbindin-D_{28K} is achieved only with calbindin-D_{28K} antisense ODN treatment (*B*). Plotted values represent the ratio of calbindin-D_{28K} content (in ng of calbindin per mg of soluble cell extract) in differentiated *versus* undifferentiated cells. Raw values (in ng) were calculated by converting the fluorescence intensity of β-galactosidase activity in the immuno-sandwich assay using the standard graph generated in the presence of purified rat kidney calbindin-D_{28K} as control antigen.

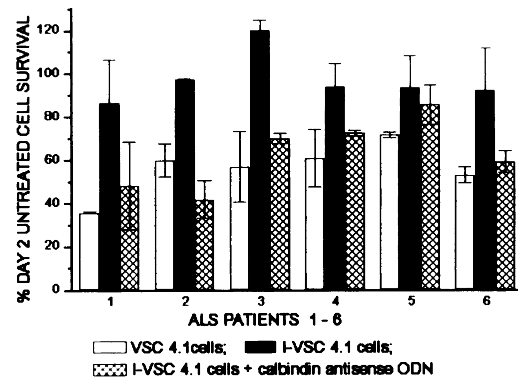

FIG. 6. Quantitative effect of immunoglobulins from patients with ALS on differentiated motoneuron hybrid cells with different levels of calbindin-D_{28K} expression. Data represent changes in cell number after a 2-day IgG exposure, expressed as a percentage of total cell number in untreated cultures. Each bar represents the mean ± SD for cell survival in described cell populations, averaging data obtained from triplicate coverslips for each experiment with each IgG. Cell counts for each coverslip were obtained from five to seven contiguous 1-mm^2 microscopic fields.

(27)] in undifferentiated VSC 4.1 cells (15) were also not influenced by the calbindin-D_{28K} gene transfection (data not shown).

Effect of Calbindin Transfection on Calcium Currents. Calcium currents in differentiated VSC 4.1 and I-VSC 4.1 cells were measured using 40 mM barium as the charge carrier, as used in our studies of the motoneuron hybrid cells (28). Since cell size and shape were variable in both VSC 4.1 and I-VSC 4.1 cell lines differentiated with cAMP, we only recorded currents from well differentiated cells with a somatic diameter of 30–40 μm to minimize differences due to cell size and shape. In both cell lines, inward currents activated near a potential of −40 mM, displayed peak amplitudes at potentials of +20 mV, and exhibited rapid voltage-dependent inactivation. No significant differences were found between the amplitudes and voltage range of activation of calcium currents in VCS 4.1 and I-VSC 4.1 cell lines (data not shown). Further studies using calcium as the charge carrier are currently in progress.

Susceptibility to ALS IgG Toxicity. Differentiated I-VSC 4.1 cells expressing high levels of calbindin-D_{28K} were significantly resistant to toxicity of ALS IgG (Fig. 6), which had been previously demonstrated to induce cell death of differentiated VSC 4.1 cells (7, 15). After 48 h, the mean survival of differentiated I-VSC 4.1 cells incubated with ALS IgG was 98.1 ± 13.1% compared to PBS-treated cultures. The same IgGs induced a 42.7 ± 13.2% cell loss in differentiated VSC 4.1 cells with very low to absent levels of immunoreactive calbindin-D_{28K} (7). Treatment of differentiated I-VSC cells for 24 h with 20 μM calbindin antisense ODN produced a markedly increased ALS IgG-mediated toxicity concomitant with a significant decrease in immunoreactivity for calbindin (Figs. 4 and 6). The mean loss of cells was 33.9 ± 15.2% in differentiated I-VSC cells treated with calbindin-D_{28K} antisense ODN. Statistical analysis of the ALS IgG-mediated toxicity by using one-way ANOVA shows a significance of $P < 0.001$ when the survival of differentiated I-VSC cells is compared with the survival of differentiated motoneuron cells with low levels of calbindin-D_{28K} expression (either differentiated VSC cells or differentiated I-VSC cells treated with calbindin-D_{28K} antisense ODN). There is no significant difference between the two differentiated cell populations in which the levels of calbindin-D_{28K} expression is very low or even absent.

DISCUSSION

The present findings demonstrate that calbindin-D_{28K} appears to influence the susceptibility of motoneuron hybrid cells to

ALS IgG-mediated toxicity. Differentiated motoneuron hybrid cells infected with retrovirus carrying calbindin-D_{28K} cDNA express high levels of calbindin-D_{28K} and are resistant to ALS IgG-mediated toxicity. Treatment of these differentiated infected cells with calbindin-D_{28K} antisense ODN significantly decreased the expression of calbindin-D_{28K} and enhanced the sensitivity to ALS IgG-mediated toxicity. We have previously documented that ALS IgG can selectively kill cells that have very low to absent levels of calcium-binding proteins (calbindin-D_{28K} or parvalbumin) (7) through a mechanism that is calcium-dependent and possibly mediated through voltage-gated calcium channels (15). ALS IgG can also increase calcium currents in motoneuron hybrid cells (28) and induces a transient increase in intracellular calcium (24) that correlates with subsequent apoptotic cell death (16).

The data obtained with our motoneuron cell line correlate well with the findings in human central nervous system where motoneurons that degenerate early in ALS have no calbindin-D_{28K} and/or parvalbumin expression whereas motoneurons that are infrequently or only very late affected have significant immunoreactivity for these calcium-binding proteins (6, 7). The importance of calcium homeostasis in the pathogenesis of motoneuron injury in ALS is supported by the *in vivo* passive transfer experiments (29), which document that injection of ALS immunoglobulins induces an increase in synaptic vesicles at motor end plates and in terminals synapsing on motoneuron perikarya as well as an increase in calcium-containing precipitates in synaptic terminals and in the cell body of motoneurons that lack calcium-binding proteins. In contrast, other neuronal populations such as dorsal horn neurons and Purkinje cells, known to be rich in calcium-binding proteins, do not show such alterations.

Our prior studies documented that the interaction of ALS IgG with neuronal-type calcium channels induces a transient increase in intracellular calcium, which leads to apoptotic cell death (15, 16, 24). Such data suggest an important role for calcium channels in this process. However, these neuronal-type (N-type, P-type, Q-type) calcium channels are not confined to motoneurons but are present on other types of neurons and cannot, *per se*, explain the selective motoneuron vulnerability. For example, ALS IgG increases P-type calcium currents in isolated Purkinje cells and in P-type channel protein reconstructed in lipid bilayers (30), but Purkinje cells are not affected in ALS. The explanation may reside in the fact that Purkinje cells possess high levels of calbindin-D_{28K} and parvalbumin (6, 7, 31). Furthermore, increased calcium current as well as increased calcium entry due to ALS IgG was also noted in neuroblastoma N18TG2 cells (24), but these cells possess high levels of calbindin-D_{28K} and are not vulnerable to ALS IgG (7). Thus, the ability of ALS IgG to interact with calcium channels and increase calcium entry may not be sufficient *per se* to induce cell death. Intracellular factors, such as calbindin-D_{28K}, could also play a critical role in cell vulnerability.

Why calbindin-D_{28K} may be neuroprotective has not been fully elucidated (32). The capacity to buffer intracellular calcium is a commonly cited function for calbindin-D_{28K}, but in differentiated VSC 4.1 cells as well as in adult mammalian motoneurons, calmodulin, another calcium-binding protein, is markedly increased and yet provides no neuroprotective effect. In a calbindin-D_{28K}-transfected pituitary cell line, calcium levels were, in fact, increased, but calcium influx through voltage-gated T- and L-type calcium channels was reduced (33). Thus the effects of calbindin-D_{28K} are not merely the consequence of calcium buffering, just as the functions of calmodulin are not merely the consequence of calcium buffering. Further evidence comes from experiments showing that in cells injected with calcium-binding proteins, calbindin-D_{28K} as well as parvalbumin significantly reduced the peak of intracellular calcium recorded for a calcium influx of an equivalent charge density in control cells (34). In these experiments, calbindin-D_{28K} not only caused an 8-fold decrease in the rate of rise in calcium but also altered the kinetics of calcium decay, suggesting a more complex role for calbindin-D_{28K} in the regulation of calcium-dependent aspects of neuronal functions. Mattson *et al.* (35) have also demonstrated that calbindin-D_{28K} containing hippocampal neurons are better able to handle increased intracellular calcium than calbindin-D_{28K}-negative neurons.

In agreement with these studies, we have used laser scanning confocal microscopy and fluo3 to quantitate intracellular calcium after exposure to ALS IgG and have noted a relatively specific transient increase in intracellular calcium lasting less than 3 min (24). The peak intracellular calcium levels after addition of ALS IgG to our differentiated motoneuron hybrid line (with absent calbindin-D_{28K} and parvalbumin) were 690 ± 70 nM. In the differentiated parental cell line (with high levels of calbindin-D_{28K} and parvalbumin), the number of cells demonstrating calcium transients was reduced to less than 20%, and in these cells the peak intracellular calcium was less than 250 nM. Baseline calcium was the same in high and low calbindin-D_{28K} and parvalbumin-containing cells. Further, the cytotoxicity of ALS IgG clearly correlated with the intracellular calcium transients (24).

The role of calbindin-D_{28K} in protecting against calcium-mediated cell death is of considerable importance since calcium may play an important role in apoptosis-associated events (36). Stable expression of proteins that buffer calcium fluxes, in particular calbindin-D_{28K}, can effectively suppress death induced by different agents in apoptosis-susceptible cells (37). The same report (37) suggests that calbindin-D_{28K} may exert its protective effect subsequent to transcriptional activation, which would be in accord with other data suggesting an active role for calbindin-D_{28K} in altering key enzymatic processes (35, 38, 39). In agreement with these findings is our demonstration that ALS IgG leads to apoptotic compromise of motoneuron hybrid cells by a calcium-dependent process (16, 24).

Thus, calbindin-D_{28K} appears to play a meaningful role in calcium homeostasis and contributes significantly to selective neuronal vulnerability. The relative lack of calbindin-D_{28K} in motoneurons may increase susceptibility to neurodegenerative processes in ALS triggered by excitotoxic damage (3, 4), antibody-induced increases in intracellular calcium (5, 15, 16, 29), oxidative injury (1, 2), alteration of trophic support (40), or disruption of the neuronal cytoarchitecture (41). Regardless of which process initiates motoneuron injury, the ability to increase calcium-binding proteins might possibly delay motoneuron injury and provide therapeutic benefit in ALS.

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