Autoantibodies to Neuronal Glutamate Receptors in Patients with Paraneoplastic Neurodegenerative Syndrome Enhance Receptor Activation

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ABSTRACT

Background: Paraneoplastic syndromes are "remote" complications of cancer characterized clinically by neurological disease. The sera and cerebrospinal fluid (CSF) from patients with paraneoplastic neurological syndromes (PNS) frequently contain autoantibodies to ill-defined neuronal antigens. We report here that neuronal glutamate receptors are targets for autoantibodies found in the serum from some patients with well-characterized PNS.

Materials and Methods: We have analyzed the serum from seven patients with well-characterized PNS for the presence of autoreactive antibodies to non-NMDA glutamate receptor subunits. Autoantibodies were assessed using Western blot, immunohistochemistry, and immunocytochemistry. Whole-cell electrophysiological recordings were used to examine the effect of antibodies on glutamate receptors expressed by cortical neurons in culture.

Results: Six of seven patients' serum contained autoantibodies to the non-NMDA glutamate receptor (GluR) subunits GluR1, GluR4, and/or GluR5/6. No patient had autoantibodies to GluR2, and only one patient exhibited weak immunoreactivity to GluR3. Electrophysiological analysis demonstrated that the serum from four of the six GluR-antibody-positive patients enhanced glutamate-elicited currents on cultured cortical neurons but had no effect on receptor function alone. Enhancement of glutamate-elicited currents was also produced by affinity-purified antibody to GluR5.

Conclusions: The occurrence of autoantibodies to specific neuronal neurotransmitter subunits in the sera of patients with PNS and the ability of these autoantibodies to modulate glutaminergic receptor function suggest that some paraneoplastic neurological injury could result from glutamate-mediated excitotoxicity.

INTRODUCTION

Paraneoplastic neurological syndromes (PNS) are a "remote" complication of systemic cancer, characterized clinically by progressive, often profound, neurological injury to focal areas in the central or peripheral nervous system in the absence of central nervous system metastases. Autoantibodies to neuronal cytoplasmic and nuclear antigens are commonly found in sera and cerebrospinal fluid (CSF) of affected patients (1).

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Three major syndromes of paraneoplastic neurological injury have been described (2,3–4,15). Paraneoplastic cerebellar degeneration associated with gynecological and breast malignancies is characterized by cerebellar loss of Purkinje cells with variable loss of other neuronal populations. These patients exhibit an antibody response, termed Type I or anti-Yo, which labels antigens of 34 and 52 or 62 kD molecular weight in Western blots of Purkinje cell concentrates. Paraneoplastic encephalomyeloneuritis is associated with small cell carcinoma of the lung and is characterized pathologically by loss of neurons in brain, spinal cord, dorsal roots, and autonomic ganglia. These patients exhibit an antibody response, termed Type IIa or anti-Hu, which produces nuclear and cytoplasmic staining of virtually all neurons and neuronal proteins of 35–42 kD in Western blots. Paraneoplastic ataxia/opsoclonus is believed to be associated with injury to brain stem omnipause neurons and is also associated with breast malignancies. This antibody response, termed, Type IIb or anti-Ri, stains nuclei and cytoplasm of virtually all neurons but, unlike Type IIa antibody response, labels antigens of 55 kD, and occasionally 70 kD, on Western blots of neuronal proteins. Each of these disorders may have its onset as long as 2 years prior to diagnosis of the underlying malignancy.

The detection of antineuronal antibodies in sera and CSF of patients with paraneoplastic neurological syndromes suggests that an autoimmune disorder may underlie these syndromes. Antibody-induced neuronal death of cultured cells in the absence of complement has supported a role for these agents in neurological injury (6), but it is not known whether Type I, IIa, or IIb antibodies are themselves contributing directly to a role in the pathogenesis of these syndromes.

We have recently reported (13) that autoantibodies to the non-NMDA neuronal glutamate receptor subunit, GluR3 (7), are present in patients with a rare form of childhood epilepsy, Rasmussen's encephalitis. In one patient, seizure frequency decreased following plasma exchange, which correlated with titers of anti-GluR3 antibodies. To explore further the occurrence of autoantibodies to GluRs in the serum of patients with other neurological diseases, we have analyzed the serum from seven patients with well-characterized paraneoplastic neurological syndromes (three with cerebellar degeneration, two with encephalomyeloneuritis, and two with opsoclonus/ataxia), for the presence of autoreactive antibodies to non-NMDA glutamate receptor (GluR) subunits. Autoantibodies were assessed using Western blot, immunohistochemistry, and immunocytochemistry of cells transfected with GluR subunit cDNAs. Whole-cell electrophysiological recordings were used to examine the effect of antibodies on glutamate receptors expressed by cortical neurons in culture. Our results support the observation that sera from some patients with paraneoplastic disease harbor autoreactive antibodies to neuronal GluRs that can modulate receptor function.

MATERIALS AND METHODS

Patient Population and Serum Sample Collection

Seven antibody-positive patients with proven paraneoplastic neurological syndromes were studied. Three patients had paraneoplastic cerebellar degeneration and Type I antibodies, two patients had paraneoplastic encephalomyelitis and Type IIa antibodies, and two patients had opsoclonus/ataxia and Type IIb antibodies (Table 1). While only one control was included in the blind study, two additional human serum samples from individuals not having PNS been tested in separate studies with the same negative results. Characterization of antibodies was carried out by immunofluorescence staining of human brain sections and confirmed by Western blot analysis as before (6) (not shown). Diagnosis of paraneoplastic disease was based upon the presence of histopathologically identified tumor tissue, the presence of autoantibodies common in paraneoplastic disease, and documentation of neuronal loss at autopsy or typical clinical symptoms and failure to detect brain metastasis during prolonged follow-up. Serum samples were collected from patients with paraneoplastic disease and controls were numbered and tested as described below. Subsequent to all procedures, samples were decoded and the results compared. Rabbit polyclonal antiserum antibodies prepared to glutamate receptor subunits were used as positive controls (4,6). All human sera and materials were acquired under guidelines approved by the Institutional Review Board, Veterans Affairs Medical Center, Salt Lake City, Utah, U.S.A. and the University of Utah.

Western Blot Analysis

Western blot analyses were conducted essentially as previously described (11–13). Bacterially

TABLE 1. Antibodies to glutamate receptor subunits in paraneoplastic neurodegenerative syndrome patient sera as revealed by immunohistochemistry, Western blot analysis, transfected cells, and electrophysiology

Patient	Antibody Type	Tumor	Pattern of GluR Receptor Staining	Western Blot Analysis	Transfected Cells	Electrophysiology
1	I	Transitional cell, bladder	R1	R5	R1, R6	Enhanced
2	IIa	Small cell, lung	None	None	None	None
3	IIb	Breast	None	None	R4	Enhanced
4	IIa	Small cell, lung	None	None	None	None
5	I	Unknown	None	None	R1(Faint R3, R6, R4)	Enhanced
6	I	Ovary	None	None	R6	None
7	IIb	Breast	None	None	R6	Enhanced
Control	None	None	None	None	None	None

overproduced portions of the non-NMDA GluR subunits GluR1 (amino acids: 658–889, termed $GluR1^{C}$; 185–521, termed $GluR1^{L}$; and 185–449, termed $GluR1^{S}$), GluR2 (175–430), GluR3 (245–451), GluR5 (233–518), and GluR6 (199–375, termed $GluR6^{S}$) were prepared, separated by SDS-PAGE and transferred to nitrocellulose filters. The filters were then blocked in phosphate buffered saline (PBS) containing 3% Carnation nonfat dry milk supplemented to 0.05% with Tween 20 (blocking solution) for 1 hr at room temperature. To each blot was added patient serum (either complete or absorbed with proteins from bacteria containing the pATH 1 vector) in blocking solution (1:400). After overnight at 4°C, blots were washed, reacted with goat anti-human alkaline phosphatase conjugated second antibody, and visualized in developer buffer (100 mM sodium chloride, 50 mM Tris (pH 9.5), 1mM $MgCl_2$) containing 0.1 mg/ml p-nitro blue tetrazolium chloride, 0.05 mg/ml 5-bromo-4-chloro-3-indolyl phosphate. The reaction was stopped with PBS containing 1 mM EDTA.

Immunohistochemistry of Rodent Brain

Immunohistochemistry of paraneoplastic sera on rodent brain was done essentially as described before (11). Mice were anesthetized with a lethal dose of anesthetic, and perfused with 25 ml of saline followed by 200 ml of 4% paraformaldehyde in PBS (pH 7.4). The brains were removed, post-fixed and cryoprotected in 4% paraformaldehyde and 25% sucrose/PBS, frozen, and cut into 25 μm-thick sections using a Zeiss microtome. The sections were washed in three changes of PBS, incubated for 1 hr in a PBS solution of 2% heat-inactivated horse serum and 0.1% Triton X-100, and incubated for 12 hr at 4°C in the immune sera diluted 1:2000 with PBS containing horse serum. The tissue was washed and incubated with peroxidase-coupled goat anti-human IgG (diluted 1:300, Jackson Laboratories) in PBS with 2% horse serum for 1 hr at room temperature, washed with PBS, and then developed to provide a peroxidase-reduced diaminobenzidine labeling.

Immunocytochemistry of Cells Transfected with GluRs

Human embryonic kidney 293 (HEK 293) cells were transfected with CMV-based mammalian expression plasmids containing the full-length cDNA for either GluR1, GluR2, GluR3, GluR4, or GluR6 as previously reported (13). Transfected cells were grown for 48 hr, fixed with 2% paraformaldehyde, blocked with PBS supplemented to 2% with horse serum with 0.2% Triton X-100, and reacted with serum as described previously (13). Typical serum dilutions ranged from 1:200 to 1:3000. As in previous studies, serum from some patients exhibited nonspecific nuclear or cytoplasmic immunoreactivity (13) that was removed by preadsorbing serum against HEK293 cells transfected with the parent expression plasmid without insert. These experiments were conducted independently three times. Im-

munoreactivity identified by the above methods was found to be diminished if serum samples were stored for prolonged times at 4°C.

Cell Culture and Electrophysiology

Neuronal cultures from fetal (E14–16) mouse cortical and hippocampal structures were prepared on poly-l-lysine coated 35 mm Falcon dishes (14). Cultures were fed every other day using a growth medium consisting of Dulbecco's modified Eagle's medium, 10% horse serum, 30 mM glucose, and 0.5 mM glutamine. Arabinosylcytosine (ARA-C) was added for 1 day during the first week in culture to suppress growth of non-neuronal cells. Electrophysiological experiments were performed on 2- to 5-week-old cultures at room temperature. External solution consisted of (in mM) 145 NaCl, 1.5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 Na-HEPES, 10 glucose, 30 sucrose at pH 7.4, 320 mOsm. Internal solution consisted of (in mM) 153 CsCl, 10 Cs-HEPES, 4 $MgCl_2$, 5 EGTA at pH 7.35, 300 mOsm. Solutions were designed for measurement of predominantly sodium inward currents and to block NMDA receptor currents when neurons are voltage clamped at −75 mV. No current was evoked by 100 mM NMDA with 1 μM glycine using this paradigm (not shown). To block other currents, 0.2-1 μM tetrodotoxin (TTX), 10 μM picrotoxin, and 100 nM strychnine were added to the external solution. External solution was perfused continuously at ≈2 ml/min. Purified sera or IgG were kept on ice until immediately before use and diluted into external solution. Ligands were applied to cells by 2 sec pressure ejection using blunt miniperfusion pipettes (≈20 μm diameter) about 100 mm from the cell. Miniperfusion pipettes were filled and the contents ejected three times with ligand solution to reduce effects of protein binding to glass. Recordings were obtained with an Axon Instruments 200A amplifier using borosilicate glass electrodes (2.5–4 megaohms). Membrane currents were filtered at 500 Hz and digitized at 2 kHz. Kainic acid, MK-801, and CNQX were obtained from Research Biochemicals International, (Natick, MA, U.S.A.), and TTX, picrotoxin, strychnine, NMDA, glycine, and salts were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.).

For electrophysiology studies, patient serum (50 μl) was diluted 1:9 in external solution buffer for electrophysiology and added to an AMICON Microcon concentrator filter unit 50 (50,000 molecular weight (MW) exclusion) and centrifuged at 10,500 rpm in an IEC microfuge until the volume was reduced to less than 100 μl (≈8 min). This procedure was repeated four times to remove small molecules such as glutamate and transfer larger serum components such as antibody into electrode buffer for application to cells. The final centrifugation was continued until the original serum volume of 50 μl was achieved. This volume was defined as 1× concentration. Serum from rabbits injected with either GluR5 fusion protein or nicotinic acetylcholine receptor subunit β2 (11–13) were used as serum controls.

Antibody specific for GluR5 was prepared from the serum of one patient by Western blot affinity chromatography as described previously (11). In brief, GluR5 fusion protein (10 μg/lane) was size fractionated by sodium dodecyl sulfate gel electrophoresis and transferred to nitrocellulose. Nitrocellulose strips containing just the transferred fusion protein were then blocked with 4% dry milk in PBS for 1 hr at room temperature. Patient serum was then added to the Western blot strips (1:1000 dilution) and they were rocked overnight at 4°C. Strips were then washed repeatedly with PBS and for 2 min with sodium glycinate elution buffer (pH 3.0) before neutralizing with 1 M sodium phosphate buffer (pH 9.0). The antibody was then concentrated by Amicon filtration (50,000 MW cut-off) and quantitated using a BioRad protein assay. Development of Western blot strips for immunoreactivity as above confirmed the removal of GluR5-specific antibody. The recovery of active affinity purified antibody was verified by repeating the Western blot analysis as described for whole serum (not shown). For electrophysiology studies, affinity-purified antibody was exchanged five times during Amicon filtration with external electrophysiology solution as above.

RESULTS

Western Blot Analysis

Western blot analysis of *trp*E-fusion proteins (see Materials and Methods) containing overlapping regions of the putative extracellular domain that precedes transmembrane 1 from GluR1, GluR2, GluR3, GluR5, or GluR6, and a domain of GluR1 ($R1^c$) that exhibits high sequence identity with other GluRs was conducted. Serum samples were prepared and tested without knowledge of sample origin. Robust immunoreactivity to GluR5 fusion protein was detected in the serum

from Patient 1 (see Fig. 1 and Table 1). Despite high sequence identity between GluR5 and GluR6, no immunoreactivity to GluR6^s was observed. The GluR6^s fusion protein is shorter than the GluR5 fusion protein with only 40% of the equivalent region of the GluR5 fusion protein represented in the GluR6^s segment. This result suggests that either autoreactivity is specific to an epitope in GluR5 in the region of GluR6^s overlap or that autoreactivity is towards GluR5 residues 377–518, which are absent from the GluR6^s fusion protein. The latter possibility is favored since these autoantibodies react with cells transfected with full-length GluR6 expressed in transfected cells (see below).

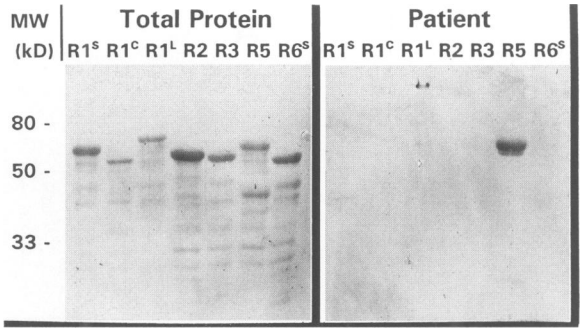

FIG. 1. Western blot analysis of serum reactivity towards glutamate receptor trpE-fusion proteins containing portions of GluR1, GluR2, GluR3, GluR5, and GluR6

The Western blot on the left shows staining of total trpE-fusion proteins. The duplicate blot on the right shows immunoreactivity of the paraneoplastic syndrome Patient 1 serum towards an equivalent Western blot and predominant immunoreactivity to GluR5. R1^S: GluR1, short construct, 185–449; R1^C: GluR1, C-terminal construct, 658–889; R1^L: GluR1, long construct, 185–521; R2: GluR2, 175–430; R3: GluR3, 245–451; R5: GluR5, 233–518; and R6^S: GluR6, short construct, 180–445. Serum was diluted 1:3000.

Immunohistochemical Analysis Employing Mouse Brain Sections

Immunohistochemical analysis of whole-serum immunoreactivity from paraneoplastic patients on free-floating mouse brain sections produced immunohistological staining of Purkinje cell cytoplasm characteristic of Type I antibody response or of neuronal nuclei and cytoplasm characteristic of Type IIa, and Type IIb antibody staining respectively (5) (not shown). The results of immunohistochemical staining from Patient 1, who exhibited strong immunoreactivity to GluR5 fusion protein (Fig. 1), were not expected. In addition to staining of Purkinje cells (Fig. 2A), this serum produced highly specific regional staining of the lateral septum, bed nucleus of the stria terminalis, central nucleus of the amygdala, and the hippocampus (Fig. 2B). Staining persisted to dilutions of 1:20,000. Compared with the staining pattern of antibodies prepared in rabbits to known non-NMDA GluR subunits (8,9,11) (Fig. 2B), the pattern corresponds closely with GluR1 (11). Staining patterns suggestive of other glutamate receptor labeling were not detected in serum from other patients or controls. Also, affinity-purified antibody to GluR5 from Patient 1 reacted poorly with brain sections and failed to produce the pattern shown in Fig. 2 (not shown). This result is discussed in greater detail in the next section.

Immunocytochemistry of HEK293 Cells Transfected with GluRs

Patient sera were reacted with HEK 293 cells transfected with GluR1, GluR2, GluR3, GluR4, or GluR6 (GluR5 does not successfully transfect in our system). Immunoreactivity to GluR1, GluR4, and/or GluR6 were found in the sera of five out of seven patients (Table 1 and Fig. 3). Serum from one out of seven patient exhibited weak immunoreactivity to GluR3 and no immunoreactivity to GluR2 was observed. Serum from Patient 1 labeled cells transfected with GluR1 or with GluR6, but not cells transfected with other GluR subunits (Fig. 3). This result suggests that in addition to GluR5, immunoreactivity is present to GluR1 and GluR6 in the serum from Patient 1 and is referred to as GluR5/6 immunoreactivity in the remaining discussion. This result also indicates that either immunohistochemical or Western blot analysis alone is not sufficient to reveal the potential complexity of GluR immunoreactivity present in the serum from patients with paraneoplastic disease.

Electrophysiology

To examine functional consequences of antibody binding to glutamate receptors, we recorded whole cell currents from fetal mouse cortical neurons in culture (14). Patient serum was prepared by Amicon filtration with filters of 50,000 MW exclusion to remove small molecules, such as glutamate, for direct application to neurons.

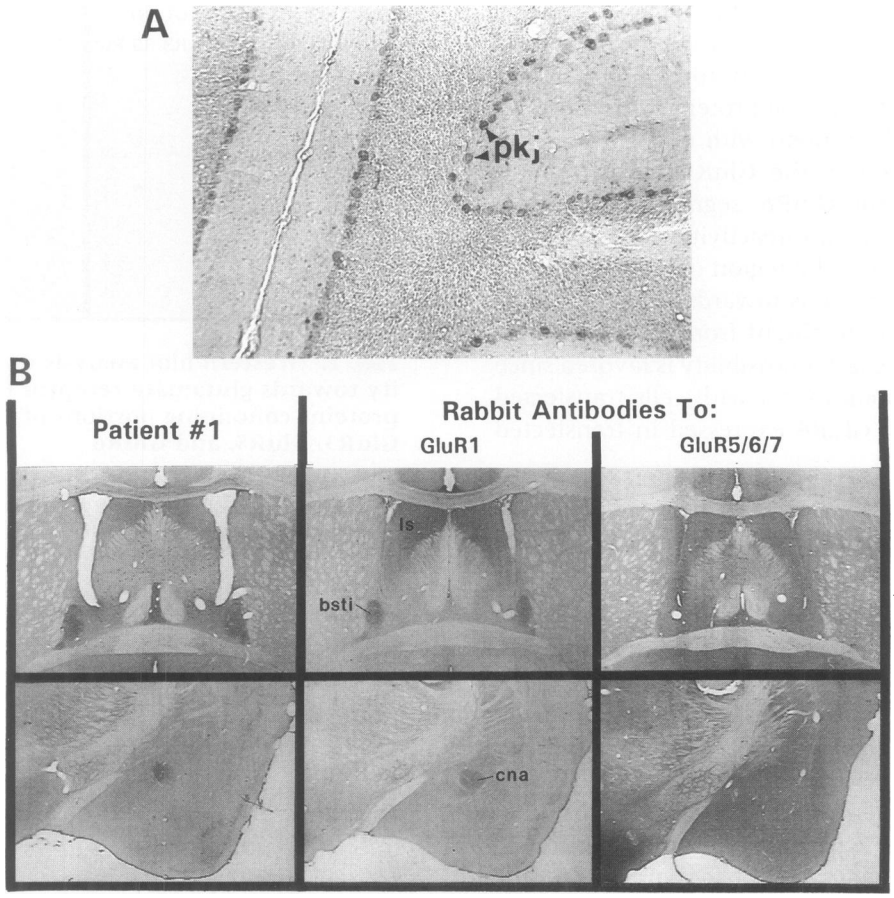

FIG. 2. Immunohistochemical pattern of staining of mouse brain regions produced by serum from PNS patient 1 and rabbit serum prepared to GluR subunits

(A) Characteristic staining of cerebellar Purkinje cells by PNS Patient 1 serum. (B) Comparison of the immunohistochemical staining of the same patient's serum in two brain regions with that of antiserum prepared in rabbits to GluR1 or GluR5/6. ls, lateral septum; bsti, bed nucleus of the stria terminalis; cna, central nucleus of the amygdala.

Coapplication of glutamate (100 μM) with sera (1:4) resulted in rapid and reversible inward currents in a subset (84%) of neurons tested (Fig. 4A). Application of the <50,000 MW filtrate did not evoke currents (not shown) indicating the absence of smaller molecules that effect receptor function such as glutamate. Average glutamate evoked whole-cell current in positively responding neurons was 1.31 ± 0.22 (mean + SEM) nanoamps (nA) with a maximum of 2.6 nA. Sera from other patients (Patients 3 [5 of 13 cells], 5 [3 of 7 cells], and 7 [3 of 5 cells]) also enhanced glutamate-induced currents (Fig. 4B and Table 1), but Patients 2 (13 cells), 4 (9 cells), and #6 (7 cells) exhibited no effect on glutamate-elicited currents (Table 1). Patient 8 was a healthy normal control in the blind study and their serum produced no effect on glutamate evoked responses (8 cells). Relative amplitudes of serum enhanced were variable between cells when compared with currents evoked by 100 μM glutamate alone. Overall, the relative enhancement of Patient 1 serum (diluted 1:4) in responding cells was 135 ± 22% (37 cells, 45–409% range, $p < 0.0001$, 2-tailed Student's t-test). Miniperfusion of sera (1:2, 1:4) alone to voltage clamped neurons did not evoke currents (7 cells). Similar experiments using human healthy control sera (Patient 8, 7 cells) did not evoke currents (Fig. 4D). Rabbit sera containing antibodies to GluR5 (1:4 dilution, 11 cells) or neuronal nicotinic acetylcholine receptor subunit β2 (1:4 dilution, 6 cells) exhibited no enhancement of glutamate evoked currents (Fig. 4D).

Patient 1 serum was selected for detailed analysis because of the robust autoimmune reactivity of serum from this patient to GluR1 and GluR5/6. Also, the strong binding of antibody

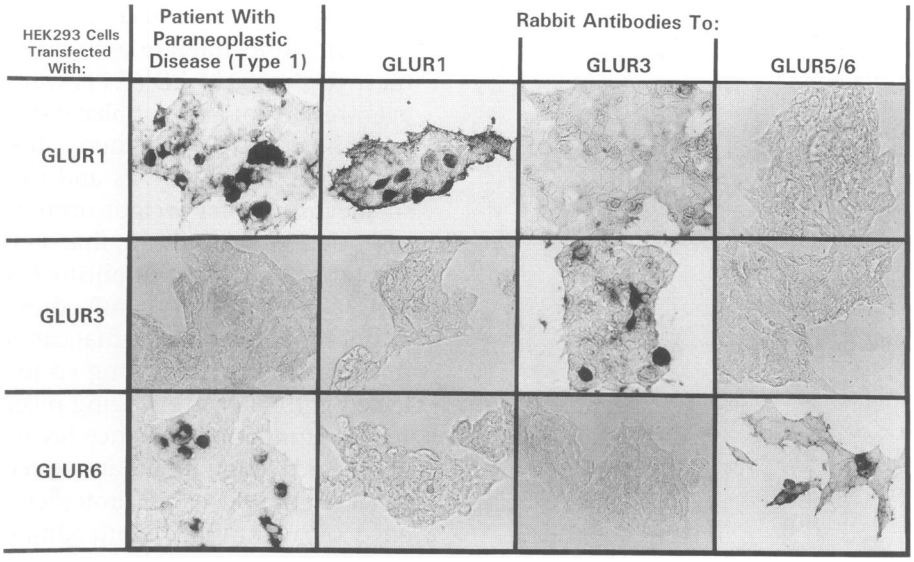

FIG. 3. Immunoreactivity of Patient 1 to human embryonic kidney cells that were transfected with either GluR1, GluR3, or GluR6

This patient exhibited staining of GluR1- or GluR6-transfected cells to dilutions of 1:4000. No staining of cells transfected with GluR3 or GluR2 and GluR4 (not shown) was observed. Rabbit polyclonal antisera prepared to the GluR subunits were used as the positive control for successful transfection. Table 1 summarizes these results for all patients studied.

from this patient to GluR5 fusion protein on Western blots (Fig. 1) provided a simple method to obtain affinity-purified antibody for electrophysiological study. Affinity purified anti-GluR5 IgG (330 ng/ml) produced enhancement of currents similar to that produced by whole serum (Fig. 4A). Peak current enhancement was observed in serial dilutions from 1:2 (107 ± 17%, range 22 to 380%, 22 cells) up to 1:512. Current enhancement was saturated at about 100% up to a dilution of 1:64 and was concentration dependent up to a dilution of 1:512 (1.3 ng/ml IgG; not shown). Enhancement of glutamate currents by serum and Western blot affinity-purified (WAC) GluR5 antibody from Patient 1 was blocked by 40 μM CNQX, a non-NMDA GluR antagonist, but was not reduced by 10 mM MK-801, an NMDA receptor blocker (not shown). To further confirm the specificity of the antibody-evoked current responses, 0.5 mg/ml of GluR2 or GluR5 fusion protein, respectively, was incubated with either Patient 1 serum or affinity-purified antibody to GluR5. The enhancement of glutamate evoked currents by serum or GluR5 affinity-purified antibody was completely blocked by incubation with GluR5 fusion protein, but incubation with GluR2 fusion protein had no effect (3 cells each, not shown).

DISCUSSION

In this study, we demonstrated that autoantibodies to neuronal glutamate receptor subunits are present in the serum of some patients with confirmed paraneoplastic neurological syndromes, including paraneoplastic cerebellar degeneration and paraneoplastic opsoclonus/ataxia. Autoantibodies were identified to GluR1, GluR4, GluR5, and/or GluR6 in five of seven patients with Type I or IIb antibody response but were not detected to GluR2. Only Patient 5 exhibited weak immunoreactivity to GluR3, which differs dramatically with the robust immunoreactivity to this subunit observed in patients with Rasmussen's encephalitis (13). Control sera exhibited no immunoreactivity to fusion proteins or transfected cells, nor were they detected in sera from two patients with paraneoplastic encephalomyeloneuritis accompanying small cell carcinoma of the lung (Patients 2 and 4, Table 1). The serum from four of seven patients enhanced the function of glutamate receptors expressed by a subset of cultured cortical neuronal cells in the presence of glutamate but had no detectable effect on receptor function in the absence of glutamate. Affinity-purified IgG specific for GluR5 from one patient enhanced glutamate receptor function in

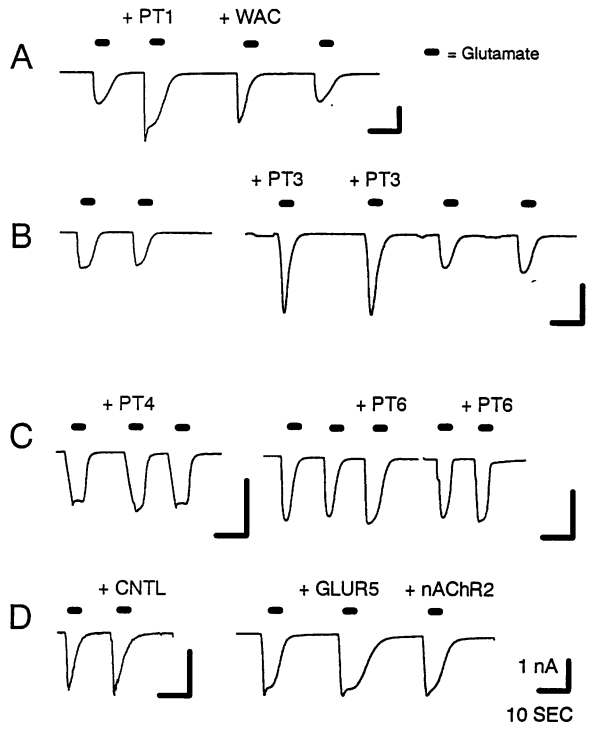

FIG. 4. Glutamate evoked whole-cell currents recorded from voltage-clamped fetal mouse cortical neurons in culture are modulated by paraneoplastic syndrome patient sera

Calibration bars are 10 sec and 1 pA throughout. (A) Glutamate (100 μM) evoked currents are enhanced by Patient 1 sera (PT1, 1:4) and by affinity-purified antibody to GluR5 (1:2) in the same neuron. (B) Glutamate receptor current enhancement was seen in a subset of neurons with other patient sera (e.g., Patient 3 [PT3], 1:4). (C) Serum from Patient 4 (PT4, 1:4) and 6 (PT6, 1:4) or (D) a control, nondiseased control patient (CNTL) did not enhance glutamate-evoked currents. Healthy human control sera and rabbit sera consisting of antibodies raised to GluR5 fusion protein or neuronal nicotinic acetylcholine receptor subunit β2 (nAChR2).

the presence of glutamate similar to serum. Serum controls from healthy individuals or serum from 3 patients that contained either no autoreactivity or autoreactivity to only GluR6 produced no effect on the electrophysiology of glutamate receptors either in the presence or absence of glutamate. Collectively, these results support the conclusion that patients with paraneoplastic cerebellar degeneration or paraneoplastic opsoclonus/ataxia may harbor highly specific autoantibodies reactive with neuronal glutamate receptor subunits in addition to other autoantibodies described previously.

Former studies have focussed on proteins that migrate with molecular weights of less than 100 kD. GluRs migrate with an apparent molecular weight of 105 kD (9,11) (not shown). However, reports of larger molecular weight antigens have been made (10) and GluRs are notably susceptible to proteolysis and the generation of smaller molecular weight proteins (11). In any case, our results indicate that studies employing Western blot or immunohistochemical methods may fail to detect autoantibodies to neurotransmitter receptor subunits that can be readily identified in studies employing cultured cells transfected with cDNAs encoding receptor proteins.

Accumulating evidence favors a role for excessive activation of glutamate receptors in neuronal death through excitotoxicity (1). The presence of autoantibodies to glutamate receptors that modulate receptor function in patients with paraneoplastic neurological syndromes may therefore be of functional significance. Subunit specificity of patient receptor autoantibodies also suggests a mechanism that would produce highly specific neuronal damage or death. For example, glutamate receptors on neurons expressing the appropriate GluR subunit(s), which are accessible to receptor-specific autoantibodies, would be subject to modulation. Enhancement of glutamate receptor activation, as would be expected in the presence of the autoantibodies identified in the present study, has been related to neuronal death through mechanisms of excitotoxicity. Consequently, only specific neurons, of specific receptor subunit composition, such as Purkinje cells that express GluR1, GluR5, and GluR6 (7), would be at risk, while other cells that do not express the appropriate subunits would be spared. Therefore, the sera of patients with two very discrete neurological syndromes (paraneoplastic cerebellar degeneration, characterized by progressive destruction of Purkinje cells, and paraneoplastic opsoclonus/ataxia), possessing autoantibodies that recognize different GluR subunits, would be predicted to affect different neurons. Similar highly specific neuronal loss or dysfunction would also be expected if antibodies affected receptor number at the cell surface or if complement-mediated lysis was involved.

This study presents the second report in which autoantibodies to neuronal glutamate receptor subunit proteins have been detected in patients with progressive neurological disease. The autoreactivity to GluR receptor subunits observed in the present study differs from the autoimmune response previously described in patients with Rasmussen's encephalitis (13) in that

sera from patients with paraneoplastic neurological syndromes recognize predominantly GluR1, GluR5, and GluR6, whereas sera from patients with Rasmussen's encephalitis react almost exclusively with GluR3. These observations raise questions as to whether antibodies to glutamate receptor subunits are present in other progressive neurological disorders as well. Examining other neurological diseases for the presence of autoantibodies to neurotransmitter receptors appears warranted.

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REFERENCES

1. Choi DW. (1992) Excitotoxic cell death. *J. Neurobiol.* **23:** 1261–1276.
2. Cunningham J, Graus F, Anderson N, et al. (1986) Partial characterization of the Purkinje cell antigens in paraneoplastic cerebellar degeneration. *Neurology* **36:** 1169–1172.
3. Graus F, Elkon KB, Cordon-Cardo C, Posner JB. (1986) Sensory neuronopathy and small-cell lung cancer: Antineuronal antibody that also reacts with the tumor. *Am. J. Med.* **45:** 45–52.
4. Greenlee JE, Brashear HR. (1983) Antibodies to cerebellar Purkinje cells in patients with paraneoplastic cerebellar degeneration and ovarian carcinoma. *Ann. Neurol.* **14:** 609–613.
5. Greenlee JE, Brashear HR, Herndon MR. (1988) Immunoperoxidase labelling of rat brain sections with sera from patients with paraneoplastic cerebellar degeneration and systemic neoplasia. *J. Neuropathol. Exp. Neurol.* **47:** 561–571.
6. Greenlee JE, Parks TN, Jaeckle, KA. (1993) Type IIa ('anti-Hu') antineuronal antibodies produce destruction of rat cerebellar granule neurons in vitro. *Neurology* **43:** 2049–2054.
7. Hollmann M, Heinemann S. (1994) Cloned glutamate receptors. *Annu. Rev. Biochem.* **17:** 31–108.
8. Huntley GW, Rogers SW, Moran T, et al. (1993) Selective distribution of kainate receptor subunit immunoreactivity in monkey neocortex revealed by a monoclonal antibody which recognizes glutamate receptor subunits GluR5/6/7. *J. Neurosci.* **13:** 2965–2981.
9. Petralia RS, Wenthold RJ. (1992) Light and electron immunocytochemical localization of AMPA-selective glutamate receptors in the rat brain. *J. Comp. Neurol.* **318:** 329–354.
10. Posner JB, Furneaux, HM. (1990) *Paraneoplastic Syndromes. Immunological Mechanisms in Neurologic and Psychiatric Disease.* Raven Press, New York.
11. Rogers SW, Hughes TE, Hollmann M, Gassic G, Deneris E, Heinemann SF. (1991) The characterization and localization of the glutamate receptor subunit, GluR1, in the rat brain. *J. Neurosci.* **11:** 2713–2724.
12. Rogers SW, Mandelzys A, Deneris E, Cooper E, Heinemann SF. (1994) The expression of nicotinic acetylcholine receptors by PC12 cells treated with NGF. *J. Neurosci.* **12:** 4611–4623.
13. Rogers SW, Andrews PI, Gahring LC, et al. (1994) Autoantibodies to glutamate receptor GluR3 in Rasmussen's encephalitis. *Science* **265:** 648–652.
14. Skeen GW, Twyman RE, White HS. (1993) The dihydropyridine nitrendipine modulates N-methyl-D-aspartate receptor channel function in mammalian neurons. *Mol. Pharmacol.* **44:** 443–444.
15. Tsukamaoto T, Yamamoto H, Iwasaki Y, et al. (1989) Antineural autoantibodies in patients with paraneoplastic cerebellar degeneration. *Arch. Neurol.* **46:** 1225–1229.

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