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Glutamate Receptor GluR3 Antibodies and Death of Cortical Cells

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Summary

Rasmussen's encephalitis (RE), a childhood disease characterized by epileptic seizures associated with progressive destruction of a single cerebral hemisphere, is an autoimmune disease in which one of the autoantigens is a glutamate receptor, GluR3. The improvement of some affected children following plasma exchange that removed circulating GluR3 antibodies (anti-GluR3) suggested that anti-GluR3 gained access to the central nervous system where it exerted deleterious effects. Here, we demonstrate that a subset of rabbits immunized with a GluR3 fusion protein develops a neurological disorder mimicking RE. Anti-GluR3 IgG isolated from serum of both ill and healthy GluR3-immunized animals promoted death of cultured cortical cells by a complement-dependent mechanism. IgG immunoreactivity decorated neurons and their processes in neocortex and hippocampus in ill but not in healthy rabbits. Moreover, both IgG and complement membrane attack complex (MAC) immunoreactivity was evident on neurons and their processes in the cortex of a subset of patients with RE. We suggest that access of IgG to epitopes in the central nervous system triggers complement-mediated neuronal damage and contributes to the pathogenesis of both this animal model and RE.

Introduction

Rasmussen's encephalitis (RE) is a rare disease characterized by progressive destruction of a single cerebral hemisphere (Rasmussen et al., 1958). The disease typically begins in the first decade of life and is manifested by severe seizures and progressive loss of functions subserved by the involved hemisphere. The underlying histopathology consists of perivascular lymphocytic cuffing, microglial nodules, neuronal destruction, and gliosis. Because the epileptic seizures are often unresponsive to conventional antiseizure drugs, surgical removal of the involved hemisphere is the standard therapy (Rasmussen and Andermann, 1989).

Recent discoveries have demonstrated that RE is an autoimmune disease in which one of the autoantigens is a glutamate receptor of the α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA) subtype, GluR3 (Rogers et al., 1994). The occurrence of epileptic seizures and inflammatory histopathology in two rabbits immunized with a GluR3 fusion protein led to the discovery of circulating anti-GluR3 antibodies in patients with active RE. The responsiveness of several patients to plasma exchange that removed circulating anti-GluR3 antibodies suggested that the anti-GluR3 gained access to the central nervous system and contributed to the neuronal degeneration (Rogers et al., 1994; Andrews et al., 1996). Anti-GluR3 antibodies isolated from ill TrpE–GluR3-immunized rabbits and patients with RE were found to have agonist properties at the AMPA subtype of glutamate receptor in mouse cortical neurons (Twyman et al., 1995). Since excessive activation of glutamate receptors can destroy cortical neurons through an excitotoxic mechanism (Choi, 1988), it seemed plausible that circulating anti-GluR3 antibodies could induce excitotoxic injury.

The goal of this work was to determine whether circulating anti-GluR3 antibodies destroyed cortical cells and if so, by what mechanism. Here, we confirm and extend our initial findings by demonstrating that a subset of rabbits immunized with a GluR3 fusion protein develops a neurological disorder that includes epileptic seizures and inflammatory histopathology. Anti-GluR3 antibodies isolated from GluR3-immunized rabbits promote destruction of cortical cells in vitro by a complement-dependent, but not excitotoxic, mechanism. The subset of GluR3-immunized rabbits with the neurological disorder exhibits abundant IgG immunoreactive-labeling of neurons and their processes in neocortex and hippocampus. The findings in the rabbit model led to studies of human tissue in which IgG and MAC-like immunoreactive labeling of neurons and their processes was discovered in the cortex of a subset of humans with RE.

Results

Repeated immunization with a glutathione-S-transferase GluR3 (GST–GluR3) fusion protein was associated with a distinctive neurological disorder in two of five rabbits. Each of the two rabbits was alert yet exhibited motor incoordination while ambulating either in an open space or in a cage. This defective motor control occurred in the absence of detectable weakness of hindlimbs or forelimbs. Responsiveness to crude sensory stimuli such as an air puff to the face or gentle pinching of extremities was intact. In addition, each rabbit exhibited occasional epileptic seizures manifested as repetitive tonic and/or clonic movements of all four extremities lasting ~20–40 s; in one animal, the repetitive clonic

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movements were immediately preceded by head nodding associated with unresponsiveness, behaviors characteristic of complex partial seizures. The epileptic seizures were followed by obtundation for several minutes prior to resumption of normal activities. The onset of this illness occurred 2 weeks after the second immunization in one animal and 3 days after the third immunization in the other. Each animal exhibited reduced food and water intake and lost ~25% of its body weight. Persistent obtundation together with weight loss eventually necessitated sacrificing both of these rabbits. No illness was evident in three other rabbits despite five to seven immunizations with GST–GluR3 over several months. Likewise, no illness was found in seven rabbits receiving multiple immunizations with GST nor in two rabbits receiving no immunizations. Inspection of hematoxylin- and eosin-stained sections revealed mononuclear cell infiltrates in circumscribed regions of cerebral cortex and surrounding blood vessels in the cortex of both cerebral hemispheres of the two sick rabbits but in none of the other twelve rabbits (data not shown), confirming our previous observations (Rogers et al., 1994).

To determine whether constituents of blood of the GST–GluR3-immunized rabbits might damage cortical cells, filtrates of plasma or serum were incubated with rat cortical cultures containing mixtures of neurons and glia. Following Sephadex G-50 chromatography to remove low molecular weight substances such as glutamate, the higher molecular weight fraction ("filtrate") was incubated with mixed neuronal-glial cultures for 24 hr, and cell death was quantitated by measurement of lactate dehydrogenase (LDH) in the media. Plasma or serum filtrates isolated from two sick and two of three healthy GST–GluR3-immunized rabbits destroyed cortical cells in a concentration-dependent manner as made evident by significant increases of LDH ranging from 3- to 5-fold over baseline (one-way analysis of variance [ANOVA], $p < 0.0001$; Figure 1A). By contrast, filtrates from seven GST-immunized rabbits and two nonimmunized rabbits induced minimal or no increases in LDH release (Figure 1A). To confirm the cell-death effect using an independent measure, a plasma filtrate from a GST–GluR3 rabbit was shown in each of three experiments to induce increased numbers of trypan blue–stained cells in comparison to a GST control (data not shown).

To test whether the cell death was excitotoxic, the effects of AMPA receptor antagonists were examined. Cell death induced by AMPA, but not the plasma filtrate, was inhibited by a noncompetitive antagonist (GYKI 52466) of AMPA receptors (Figure 1B). Likewise, a competitive antagonist of AMPA and kainate receptors, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), inhibited cell death induced by AMPA but not the plasma filtrate (data not shown). Cell death was not inhibited by either a competitive (100 μM D-APV) or a noncompetitive (MK-801, 1 or 10 μM) antagonist of the N-methyl-D-aspartate (NMDA) receptor (data not shown); cytotoxicity experiments routinely included 100 μM D-APV to exclude any LDH release due to NMDA receptor activation. Because it is possible that a receptor could be activated by an allosteric mechanism insensitive to CNQX or GYKI 52466, the presence of excitatory properties of these

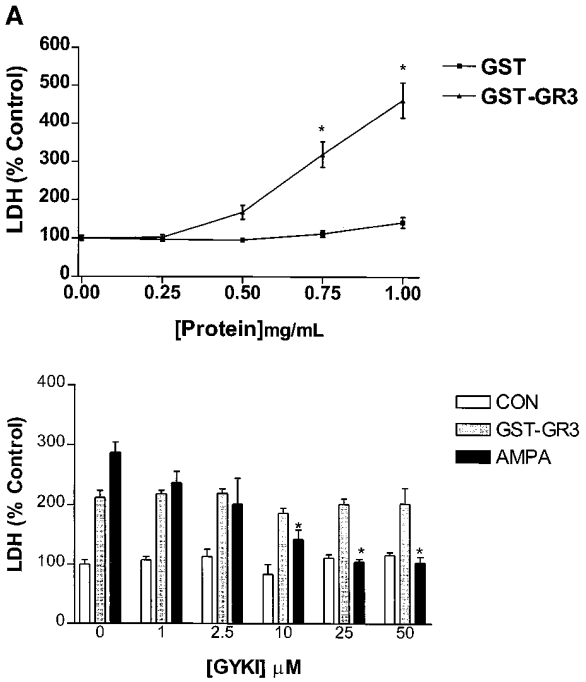

Figure 1. Cytotoxicity of Plasma Filtrates of GST–GluR3-Immunized Rabbits

(A) Protein isolated from serum or plasma of GST–GluR3-immunized rabbits induces release of LDH from cultures of mixed neurons and glia in a concentration-dependent manner. The plot shows the mean ± SEM of LDH released following 24 hr exposure of cortical cells to varying concentrations of serum or plasma collected from five GST–GluR3-immunized and nine control (seven GST-immunized and two nonimmunized) rabbits (3–6 wells/animal). Data were analyzed by one-way analysis of variance (ANOVA) followed by post hoc Tukey-Kramer multiple comparisons test, which disclosed GST–GluR3 at 0.75 mg/ml compared to no additions, $p < 0.001$ as denoted by *; GST–GluR3 at 1.0 mg/ml compared to no additions, $p < 0.001$ as denoted by *; and GST at 1.0 mg/ml compared to no additions, $p > 0.05$. The amount of LDH due to LDH present in the plasma added at a concentration of 1 mg/ml was less than 4 units/l and did not differ between GST–GluR3 and control. Control wells typically have 20–30 units/l of LDH. The amount of protein in the plasma of these rabbits was ~40 mg/ml; therefore, plasma added at 1 mg/ml to the media represents a dilution of ~1:40.

(B) An experiment demonstrating that the noncompetitive antagonist GYKI 52466 inhibits cell death induced by AMPA (10 μM) but not by 1 mg/ml of plasma filtrate from an ill GST–GluR3-immunized rabbit. Bars represent mean ± standard error of 3–12 wells per condition. Control (con) wells contained MEM and received only the indicated concentrations of GYKI. Similar results were obtained in experiments with each of the four GST–GluR3-immunized rabbits whose plasma filtrates destroyed cortical cells. There was significant inhibition of AMPA toxicity by GYKI 52466 as compared to AMPA in the absence of GYKI 52466 ($p < 0.001$ as denoted by *, one-way ANOVA).

plasma filtrates was examined directly in whole-cell recordings of cultured rat cortical neurons. Plasma filtrates isolated from the two sick rabbits were applied to fourteen neurons at a concentration of 1 mg/ml and to nine neurons at a concentration of 6 mg/ml; in contrast to robust currents evoked by kainic acid (300 μM) in each neuron (range, 0.66–13.7 nA; mean, 4.40 nA), no detectable currents were evoked by plasma filtrate (Figure 2). Bath application of cyclothiazide (100 μM), a compound that inhibits AMPA receptor desensitization,

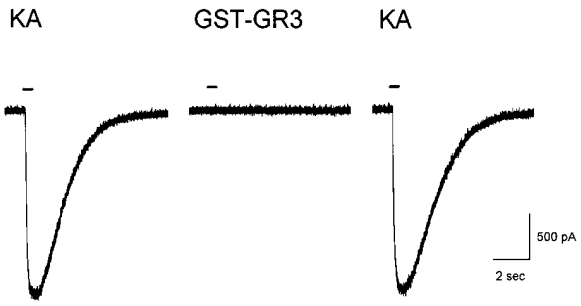

Figure 2. Kainate, but Not Plasma Filtrate, Activates Inward Current in Cortical Neuron

A representative experiment demonstrating the inward current in a rat cortical neuron in vitro evoked by kainic acid (300 μM) but not by plasma filtrate (6 mg/ml) isolated from a GST–GluR3-immunized rabbit. Horizontal bars denote drug or filtrate applications (0.5 s). Equivalent results were obtained with other preparations of filtrates and IgG as described in the text.

enhanced the kainic acid response but failed to reveal any responses to plasma filtrates in five cells tested (data not shown). Although devoid of detectable excitatory properties, cytotoxic effects were readily demonstrable in other aliquots of these plasma preparations at concentrations ranging from 0.5–1.0 mg/ml (Figure 1A) and in an aliquot isolated from the same tube containing the sample used for electrophysiologic testing in Figure 2. To further test for excitatory effects in a more homogeneous population of neurons expressing AMPA receptors, the effects of IgG isolated from serum of the two sick rabbits (see below) were examined in neurons cultured from the rat dentate gyrus; although responses to kainic acid (range, 72–795 pA; mean, 270 pA) were readily found, no detectable responses to IgG (0.2–2.0 mg/ml) were obtained in any of eight neurons recorded in the absence or presence of cyclothiazide (data not shown). The presence of GluR3 in these cells was demonstrated by identification of a band of appropriate size on an immunoblot prepared from homogenates of cultured cortical cells and probed with a peptide affinity-purified antibody specific for GluR3 (Wenthold et al., 1996; data not shown). As demonstrated in immunocytochemical experiments described below, the majority of GluR3 in these cultures is present on neurons. The absence of neuroprotective effects of AMPA receptor antagonists, together with the absence of detectable excitatory effects of the plasma filtrates or IgG, argues that the mechanism of cell death is not excitotoxic.

To determine whether the cell death was mediated by antibodies of the IgG type, the IgG fraction was separated from the remainder of the plasma by Protein G affinity chromatography. The effectiveness of the Protein G column was assessed by probing immunoblots prepared from aliquots of the IgG fraction and Protein G filtrate with anti-IgG and anti-IgM antibodies; the vast majority of the IgG and IgM was found to reside in the IgG fraction and Protein G filtrate, respectively (data not shown), thereby demonstrating the selectivity of the Protein G chromatography for IgG. In contrast to the plasma filtrate itself, neither the IgG fraction nor the Protein G filtrate was sufficient to induce LDH release

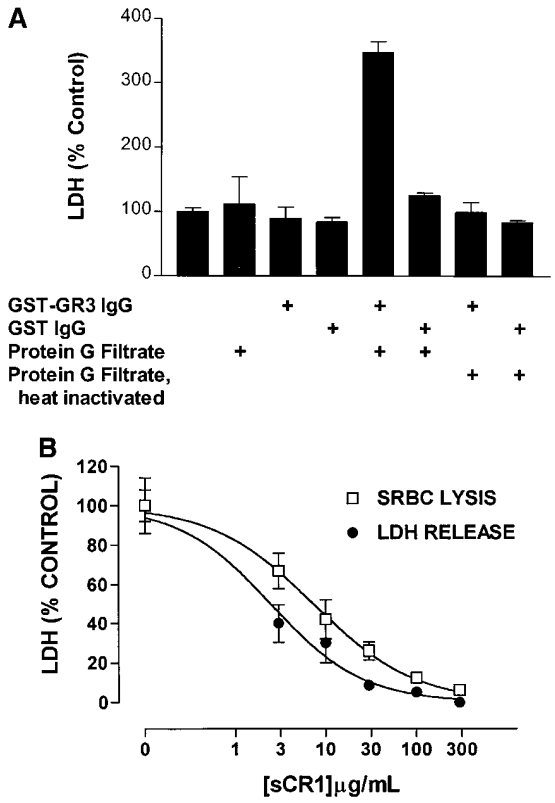

Figure 3. IgG and Complement Are Required for Cytotoxicity

(A) Representative experiment demonstrating that IgG is necessary but not sufficient to cause cell death and that the plasma fraction required to reconstitute the IgG-mediated cell death can be inactivated by heat. Cortical cells were incubated with IgG (340 μg/ml) isolated by Protein G affinity chromatography and/or filtrate (1.3 mg/ml) from the Protein G column. Filtrate refers to that portion of the rabbit plasma that was not retained by the Protein G affinity column. Heat inactivation was produced by a 30 min incubation at 56°C. Heat inactivation eliminated the cell death–producing effects of IgG isolated from each of the four GST–GluR3-immunized rabbits whose plasma or serum filtrates destroyed cortical cells.

(B) Representative experiment demonstrating that addition of sCR1 inhibits lysis of sheep red blood cells (SRBC) as well as the cytotoxic effects of rabbit sera on cortical cells in vitro in a concentration-dependent manner. Lysis was induced by incubating rabbit serum with SRBC coated with anti-Forssman's antibody; cytotoxic effects of the plasma fraction from a GST–GluR3-immunized rabbit were measured as in Figure 1A. To facilitate comparison of sCR1 in these two assays, results of each assay were normalized and expressed as percentage of control in absence of sCR1. Values represent mean ± SEM of four replicates at each point. Additional experiments demonstrated that sCR1 inhibits the cytotoxic effects of the IgG fraction mixed with the filtrate from the Protein G affinity column (data not shown).

(Figure 3A). Mixing the two together, however, reconstituted the cell-death effects, demonstrating that the IgG was necessary but not sufficient to produce cell death. The absence of cytotoxic effects of the Protein G filtrate argues against IgM mediating the cytotoxic effects.

To further characterize the serum antibody response, the presence and titers of both IgG and IgM antibodies labeling recombinant TrpE–GluR3 or TrpE itself were examined by immunoblotting at dilutions of 1:500 and 1:3000. Serum from each of the five animals immunized with GST–GluR3 exhibited IgG immunoreactivity to

TrpE–GluR3 at a dilution of 1:3000, although the intensity of the immunoreactivity varied markedly among different animals; none of these sera exhibited immunoreactivity to TrpE. No immunoreactivity to TrpE–GluR3 was detected in serum of GST-immunized or nonimmunized animals (data not shown). There was no correlation between the relative immunoreactivity of the anti-GluR3 IgG measured by immunoblot and either the cytotoxic effects or the presence of disease among the five GST–GluR3-immunized rabbits. In contrast to the results of the anti-GluR3 IgG, only very weak IgM immunoreactivity to TrpE–GluR3 was detected and only at dilutions of 1:500; this immunoreactivity was present in the serum of a subset of both GST–GluR3 and GST-immunized animals (data not shown).

The above results suggested that components of the filtrate cooperated with the IgG to cause cell death. To determine whether IgG produced cell death by a complement-dependent mechanism, the effects of heat inactivation were examined because of the exquisite sensitivity of complement to this treatment (Abbas et al., 1991). Preincubation of the plasma fraction devoid of IgG at 56°C for 30 min eliminated its cytotoxic effects when mixed with IgG (Figure 3A), a finding consistent with complement-mediated cytotoxicity. To further assess the potential role of complement, the effects of soluble complement receptor 1 (sCR1) on plasma filtrate–induced cell death were examined. CR1, a member of the regulator of complement activation family (Weisman et al., 1990), is a membrane protein expressed on erythrocytes and lymphocytes; it suppresses complement activation by reversibly binding to complement proteins C3b and C4b. sCR1 is a recombinant form of CR1 lacking the transmembrane and cytoplasmic domains; sCR1 inhibits complement activation in vitro and in vivo (Weisman et al., 1990). To monitor the efficacy of sCR1, a standard assay for complement lysis was employed as described in the Experimental Procedures. sCR1 inhibited both complement-dependent lysis of sheep red blood cells and the cytotoxic effects of serum filtrates in cortical cultures in a concentration-dependent manner with similar potencies (Figure 3B). Additionally, the reconstitution of cell death using IgG and Protein G filtrate from a GST–GluR3-immunized rabbit was inhibited by sCR1 (data not shown).

To determine whether the IgG fraction producing the cell death consisted of anti-GluR IgG in particular, the effects of selective removal of anti-GST and anti-GluR IgG on the cytotoxic effects of plasma filtrate were examined. Preabsorption of the plasma filtrate with either a GST–GluR3 (Figure 4A, right, lane 1) or a GST (Figure 4A, center, lane 1) affinity column removed the majority of IgG-recognizing GST. Preabsorption of the plasma filtrate with a GST–GluR3 (Figure 4A, right, lane 2) column virtually eliminated IgG-recognizing GluR, whereas preabsorption with a GST (Figure 4A, center, lane 2) column resulted in minimal reduction of IgG-recognizing GluR. The plasma filtrate alone produced robust cytotoxicity as made evident by a 200% increase of LDH release (Figure 4B). The cytotoxic effects were virtually abolished in the fraction from which IgG-recognizing GluR was virtually eliminated, whereas robust cytotoxic effects persisted despite removal of the majority of IgG-recognizing GST. Together with experiments demonstrating that IgG is necessary for the cytotoxicity (Figure 3A), the elimination of the cytotoxic effects in fractions from which IgG-recognizing GluR was selectively removed demonstrates that anti-GluR IgG in particular is required for the cytotoxic effects of the plasma filtrate.

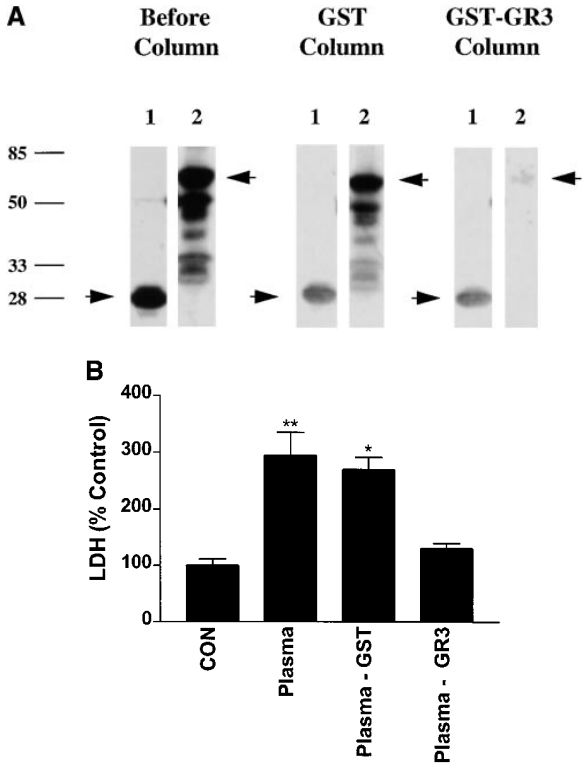

Figure 4. Anti-GluR Is Required for Cytotoxicity

(A) Immunoblots of plasma from GST–GluR3-immunized animal before affinity chromatography (left), after preabsorption with GST column (center), or after preabsorption with GST–GluR3 column (right). In each panel, the following proteins (approximate molecular weights in kilodaltons) were loaded into the respective lanes: lane 1, GST protein (26 kDa; denoted by arrows to the left of lane 1) and lane 2, TrpE–GluR3 (60 kDa; denoted by arrows to the right of lane 2; Rogers et al., 1994). Preabsorption with the GST column is associated with marked reduction of IgG-recognizing GST (26 kDa; center, lane 1) but minimal reduction of IgG-recognizing TrpE–GluR3 (60 kDa; center, lane 2). Preabsorption with the GST-GR3 column is also associated with marked reduction of IgG-recognizing GST (26 kDa; right, lane 1) but, in contrast to the GST column, with virtual elimination of IgG-recognizing TrpE–GluR3 (60 kDa; right, lane 2). Bands evident in lane 2 that migrate more rapidly than TrpE–GluR3 likely represent degradation products of TrpE–GluR3, because these are removed by GST–GluR3 affinity column. These immunoblots were probed with plasma filtrates from one of four animals subjected to affinity chromatography and presented in experiments in Figure 4B and are representative of results obtained with plasma filtrates of all four animals.

(B) LDH release induced by the plasma filtrates of 4 GST–GluR3-immunized rabbits (1 mg/ml) before or after preabsorption with GST or GST-GR3 affinity column as shown in immunoblots in (A). Control (CON) refers to LDH released following incubation with MEM. Data were analyzed by one-way ANOVA followed by post hoc Tukey-Kramer multiple comparisons test, which disclosed plasma compared to plasma-GR3, $p < 0.001$ as denoted by ** and plasma-GST compared to plasma-GR3, $p < 0.01$ as denoted by *.

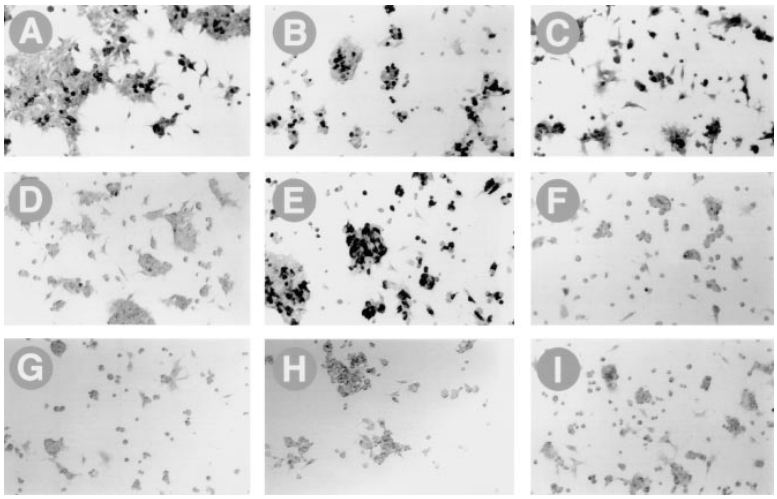

Figure 5. Antisera Label GluR3- but Not GluR1- or GluR6-Transfected HEK Cells

Immunocytochemistry of human embryonic kidney cells transiently transfected with GluR1 (Figures 5A, 5D, and 5G), GluR3 (Figures 5B, 5E, and 5H), or GluR6 (Figures 5C, 5F, and 5I). Top panel shows positive controls in which cells transfected with designated subunits were incubated with antisera raised against fusion proteins containing sequences from analogous portions of GluR1 (Figure 5A) or GluR6 (Figure 5C); GluR3 (Figure 5B) was detected with a peptide affinity-purified antibody (0.16 μg/ml) raised against peptide containing residues 372–395 of GluR3. Middle panels show that serum from an ill rabbit with cytotoxic IgG exhibits immunoreactivity for GluR3- (Figure 5E) but not GluR1- or GluR6-transfected (Figures 5D and 5F, respectively) cells. Bottom panels demonstrate no detectable immunoreactivity for any of the subunits with sera from a GST-immunized rabbit. Unless otherwise specified, all primary antisera were used at a dilution of 1:1000. Methods of detection using biotinylated anti-rabbit secondary antibodies were followed as previously described (Rogers et al., 1994).

To determine the specificity of the anti-GluR IgG for GluR3 in comparison to other closely related members of the GluR family, human embryonic kidney cells (HEK 293) transiently expressing GluR1, GluR3, or GluR6 were examined for immunoreactivity with sera isolated from GST–GluR3-immunized animals. Sera from the three GST–GluR3-immunized animals exhibiting the most marked cytotoxic effects in the experiments portrayed in Figure 1A were tested. Labeling of HEK cells transfected with GluR3 was readily detected, whereas no labeling of cells transfected with GluR1 or GluR6 was found (Figure 5). These findings underscore the specificity of the anti-GluR IgG for GluR3 in particular.

The finding that anti-GluR3 IgG sera are specific for GluR3 implies that these cytotoxic antibodies exert their toxic effects by binding to cells expressing GluR3. Since the cortical cultures contain a diversity of cell types, including neurons and astrocytes, we sought to determine the cell type(s) expressing GluR3 in these cultures. To this end, immunocytochemistry was performed using an affinity-purified antibody raised against a peptide sequence unique to GluR3 (residues 373–395 as numbered by Keinanen et al., 1990), and the cellular locale of the GluR3 immunoreactivity was assessed by coincubation with either a neuron- or astrocyte-specific marker (anti-MAP2 [microtubule-associated protein 2] and anti-GFAP [glial fibrillary acidic protein], respectively). These double-labeling experiments revealed that the vast majority of cells labeled with anti-GluR3 were also labeled with anti-MAP2 but not with anti-GFAP (data not shown). Only a minority of cells labeled with anti-GluR3 were labeled with anti-GFAP, and such cells were lightly labeled (data not shown). These findings are consistent with previous findings of Eshhar et al. (1993) and indicate that the majority of GluR3 expressed in these culture conditions resides on neurons.

The deleterious effects of plasma-derived GluR3 IgG on cortical cells in vitro suggested that GluR3 IgG in the systemic circulation might contribute to the neurological disease observed in two of the rabbits in vivo. However, the correlation between the deleterious effects in vitro and the disease was incomplete. Among GST–GluR3-immunized rabbits, cell death was induced by plasma GluR3 IgG from the two sick rabbits but also from two of three apparently healthy rabbits. If the GluR3 IgG in the peripheral circulation contributes to the disease, it must gain access to GluRs in the central nervous system. Normally, the blood–brain barrier limits access of circulating IgG to the central nervous system (Brightman et al., 1970), the site of most GluRs. We therefore asked whether IgG was detectable in the brains of any of the GST–GluR3-immunized or control rabbits. Immunocytochemical analyses disclosed the presence of robust IgG-like immunoreactivity in neocortex (Figure 6B) and hippocampus but not thalamus of the two sick rabbits; the IgG-like immunoreactivity labeled a subset of neuronal cell bodies in deep cortical layers and their neurites (Figure 6B). By contrast, IgG-like immunoreactivity was virtually undetectable in cortex and hippocampus of the three healthy GST–GluR3-immunized rabbits (Figure 6A) and in the nine control rabbits (data not shown). Although present at low or undetectable levels in cortex, IgG-like immunoreactivity is present in the pia mater of otherwise normal animals (Fabian and Ritchie, 1986; Schmidt-Kastner et al., 1993) and was evident in all fourteen rabbits studied here (data not shown).

To verify that this IgG-like immunoreactivity was indeed IgG, cryostat sections from ill and healthy GST–GluR3-immunized rabbits were homogenized and analyzed by PAGE. Immunoblots (Figure 7A) probed with an affinity-purified antiserum raised against rabbit IgG demonstrated increased labeling of a 50 kDa band in cortical homogenates from a sick GST–GluR3-immunized rabbit (Figure 7A, lane 2) in comparison to a healthy GST–GluR3 animal (Figure 7A, lane 3). The similarity in size to pure IgG (Figure 7A, lane 1) suggests that this 50 kDa band is the heavy chain of IgG and, together with the immunocytochemical results, supports the idea that increased amounts of IgG are present in the brains of the sick rabbits. No IgM was detectable in cortical homogenates of any of these animals as assessed by

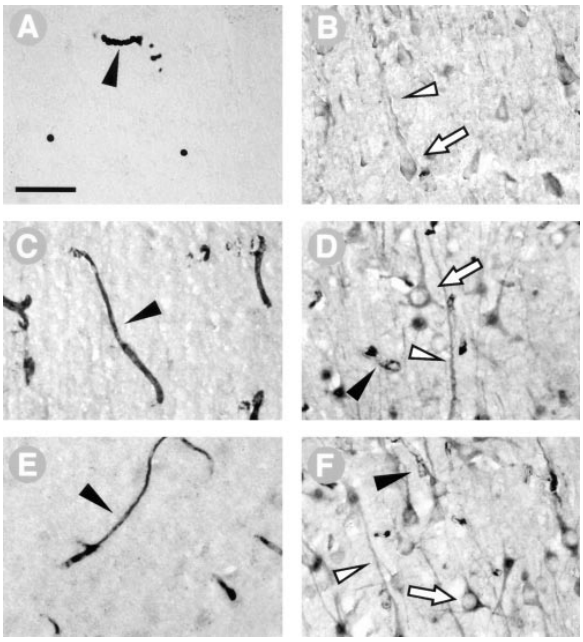

Figure 6. Immunohistochemistry of Rabbit and Human Cortex

(A and B) Staining of brain samples from healthy and sick GST–GluR3-immunized rabbits using an anti-rabbit IgG antibody (1:60,000).

(A) No cellular labeling was seen in the brains of healthy rabbits. Red blood cells (closed arrowhead) present in capillaries were stained due to endogenous peroxidase activity; the pial surfaces of these brains were also labeled (data not shown), as has been reported previously (Fabian and Ritchie, 1986).

(B) By contrast, a subset of neurons in deep cortical layers (shown) and hippocampi (data not shown) of the sick rabbits was prominently labeled (open arrow). Note staining of the apical dendrites of these neurons (open arrowhead). In control experiments, omission of the anti-rabbit IgG antibody eliminated all labeling.

(C–F) Staining of brain samples from patients with RE and complex partial epilepsy using antibodies directed at human IgG (1:1000, [C] and [D]) and MAC (1:2000, [E] and [F]).

(C and E) Images of the staining obtained with one of the complex partial epilepsy patients, representative of the group of patients lacking neuronal labeling with these antibodies (one RE and three complex partial epilepsy cases). Anti-IgG (C) and anti-MAC (E) labeled only capillaries (closed arrowheads), presumably reflecting, respectively, the presence of circulating human immunoglobulins and nascent MACs that may have formed prior to fixation of tissue.

(D and F) Representative photographs of sections from one of the two RE samples demonstrating positive neuronal labeling with anti-human IgG (D) or anti-MAC (F) antibodies. Identical patterns of labeling were obtained with each antibody, including robust staining of neurons (open arrows) and their apical dendrites (open arrowheads). Each antibody labeled subsets of neurons in discrete patches scattered about the sections, and the domains stained by the anti-MAC antibody generally overlapped with those labeled by the anti-IgG antibody. Capillaries were darkly stained as well (closed arrowheads). Omission of the primary antibody or preincubation of the primary with the antigen against which it was raised, but not an irrelevant protein, abolished all cellular and the majority of capillary labeling, confirming specificity of the staining shown here. Scale bar in (A) is 50 μm and pertains to all photographs. The top of each photo is parallel to the pial surface.

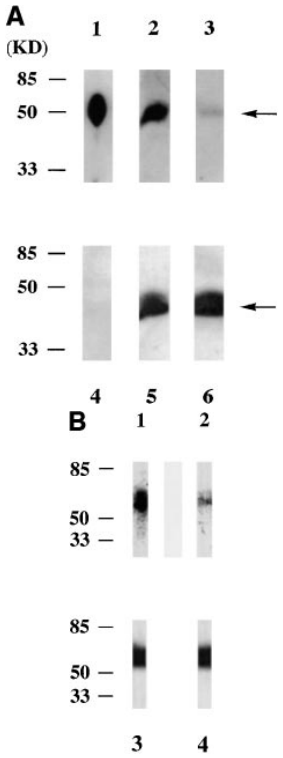

Figure 7. Immunoblots Disclose Increased IgG and anti-GluR3 in Cortical Homogenates of Ill Rabbits

(A) To confirm the presence of increased IgG immunoreactivity in the cortex of an ill GST–GluR3-immunized rabbit compared to a healthy GST–GluR3-immunized rabbit, 200 μg aliquots of homogenates of six 30 μm sections of cortex were subjected to PAGE and immunoblots were incubated with the anti-rabbit IgG antisera used for immunohistochemistry (lanes 1, 2, and 3). Lane 1 contains 0.1 μg purified rabbit IgG; extracts are from cortical sections of an ill (lane 2) or healthy (lane 3) GST–GluR3-immunized rabbit; note the greater immunoreactive labeling of the band (arrow) approximating 50 kDa (which likely corresponds to the heavy chain of IgG) from the ill (lane 2) compared to the healthy (lane 3) rabbit. To control for loading and transfer, this blot was stripped and reprobed with an antibody to β actin (lanes 4, 5, and 6), which showed similar immunoreactive labeling (arrow) for the ill (lane 5) and healthy (lane 6) rabbits.

(B) To test for the presence of anti-GluR3 in rabbit brain, TrpE–GluR3 and TrpE (data not shown) were subjected to PAGE, transferred to Immobilon, and incubated with extracts from homogenized cryostat brain sections of an ill GST–GluR3- and a healthy GST–GluR3-immunized rabbit. IgG was detected by incubation of the blot with ^{125}I-Protein A followed by autoradiography; note the presence of increased immunoreactive labeling of TrpE–GluR3 with the extract from the ill (lane 1) compared to the healthy (lane 2) rabbit. To control for loading and transfer, this blot was stripped and reprobed with a peptide affinity-purified antibody specific to GluR3; immunoreactive bands of similar intensity with size approximating 60 kDa were found for the lanes probed from the ill (lane 3) and healthy (lane 4) rabbits. The absence of immunoreactive labeling of TrpE detected in an immunoblot with the cortical homogenate from the ill GST–GluR3 rabbit (data not shown) demonstrates that the immunoreactivity in lane 1 is directed to GluR3.

stripping and reprobing this immunoblot with an anti-IgM (data not shown). The strength of association between the neurological disease and the presence of increased amounts of IgG in the cortex was measured with a nonparametric test of association, the Spearman rank order correlation test; this analysis revealed a significant association ($p < 0.0005$) between the two variables.

To determine whether the IgG included anti-GluR3 IgG, immunoblots with both TrpE and TrpE–GluR3 were incubated with extracts of cortical homogenates isolated from a sick and a healthy GST–GluR3-immunized

rabbit; the presence of immunoreactivity for TrpE–GluR3 but not TrpE (TrpE not shown) in the GST–GluR3 cortical homogenates (Figure 7B, lane 1) demonstrates the presence of increased amounts of anti-GluR3 IgG in the brain of the sick compared to the healthy GST–GluR3-immunized rabbit.

The presence of robust IgG immunoreactive labeling of neurons in the cortex of sick but not healthy rabbits led us to query whether similar evidence of neuronal IgG deposition was detectable in humans with RE. Additionally, the availability of antibodies directed against components of the human complement system permitted a search for evidence of complement activation with an antibody directed against the MAC; the lack of reagents effective for immunohistochemical labeling of rabbit complement proteins in our hands precluded analogous studies of rabbit brain. Immunohistochemistry using antibodies against IgG and MAC was performed in parallel on 30 μm sections of cortex surgically resected from three RE and three complex partial epilepsy cases and from cortex obtained at autopsy from one Alzheimer's disease (AD) patient. The AD tissue served as a positive control, as several investigators have found complement (but not IgG) deposition in the brains of AD patients (e.g., McGeer et al., 1989), an observation confirmed in the present study (data not shown). The complex partial epilepsy samples served as negative controls. Staining with both the anti-IgG and anti-MAC antibodies revealed robust labeling of neuronal cell bodies and apparent apical dendrites in two of the three RE cases studied (Figures 6D and 6F, respectively). Staining with each antibody typically labeled the perimeter of the cell body and excluded the nucleus; however, staining of nuclei was evident in a subset of apparent neurons that exhibited a shrunken pyknotic appearance. Cellular staining of the RE brains was detected on subsets of neurons contained within discrete areas of ~300–2000 μm scattered about the sections; this multifocal pattern is similar to previous descriptions of neuronal loss and inflammatory cell infiltrates in RE brains (Farrell et al., 1995). Importantly, each antibody labeled neurons in apparently overlapping patches of adjacent or nearby sections. In contrast to these findings, no distinct cellular labeling was noted with either the anti-IgG or anti-MAC antibodies in one of the three RE cases or in any of three complex partial epilepsy cases (Figures 6C and 6E, respectively). Omission of the primary antibody eliminated distinct cellular labeling in all samples. Preincubation with the proteins against which the antibodies were raised (human IgG and soluble MAC for anti-IgG and anti-MAC, respectively) also eliminated distinct cellular labeling and the majority of capillary staining, whereas preincubation with irrelevant proteins (rabbit IgG and C4, respectively) did not affect staining of cells or capillaries (data not shown). Together, these control experiments support the specificity of staining observed with these antibodies.

Discussion

Four principal findings emerge from this work. First, a subset of GST–GluR3-immunized rabbits developed a neurological disorder characterized by epileptic seizures and motor incoordination. Second, anti-GluR3 isolated from GST–GluR3-immunized rabbits promoted death of cortical neurons in vitro by a complement-dependent, but not excitotoxic, mechanism. Third, the subset of GST–GluR3-immunized rabbits with the neurological disorder exhibited abundant IgG immunoreactivity decorating neurons and their processes in neocortex and hippocampus. Fourth, a subset of patients with RE exhibited both IgG-like and MAC-like immunoreactivity decorating neurons and their processes in cortex.

The present results confirm and extend our original findings (Rogers et al., 1994), in that epileptic seizures and inflammatory cell infiltrates were evident in two of five rabbits immunized with a GST–GluR3 fusion protein (including residues 246–455) that closely resembles the GluR3 sequence of the TrpE–GluR3 protein (residues 246–458) used in the initial study. This neurological syndrome is almost certainly triggered by the GluR3 protein itself, because a similar syndrome did not occur in any of seven controls immunized with GST alone; moreover, eliciting the same syndrome with distinct bacterial proteins (TrpE in the original study and GST in the present study) fused to GluR3 underscores the pivotal nature of GluR3. The absence of this distinctive neurological syndrome in more than 50 rabbits immunized with TrpE fusion proteins containing GluR1, GluR2, GluR5, or GluR6 or neuronal nicotinic acetylcholine receptor (nAChR) subunits in our initial study (Rogers et al. 1994) suggests that sequences unique to GluR3 are critical, a suggestion to be further tested in future studies. The abnormalities in these rabbits recapitulate several features of RE, including epileptic seizures, inflammatory histopathology, and selectivity of the anti-GluR antibodies for GluR3 but not the closely related subunits GluR1 or GluR6. The disorder in the rabbits differs from RE in the absence of overt weakness or unilateral neurologic deficit and in the bihemispheric distribution of the inflammatory cell infiltrates. The features common to these disorders provide hope that study of the rabbits will facilitate insights into the pathogenesis of the disease in humans.

The responsiveness of patients with RE to plasma exchange (Rogers et al., 1994; Andrews et al., 1996) that removed circulating anti-GluR3 (Rogers et al., 1994) suggested that these antibodies may contribute to the progressive neuronal loss and hemispheric atrophy of this disease. Glutamate receptors are pivotally involved in excitotoxicity, a mechanism of cell death in the mammalian central nervous system whereby excessive activation of these receptors by glutamate can destroy cells (Choi, 1988). The report that anti-GluR3 antibodies isolated from the ill TrpE–GluR3-immunized rabbits and two patients with RE can activate CNQX-sensitive currents in cortical neurons (Twyman et al., 1995) raised the possibility that the antibodies may destroy cells through an excitotoxic mechanism. Despite replicating the neurological disorder observed in our initial report, no evidence of any currents activated by either filtered plasma or IgG isolated from serum of the ill rabbits was detected in recordings from cortical or dentate gyrus neurons in the present study. Multiple differences exist between the experimental protocols of Twyman et al. (1995) and the present study, including (1) the neurons

studied (E14–E16 mouse cortex studied at 4–6 weeks in vitro versus E18 rat cortex studied at 2–3 weeks in vitro or P4 rat dentate gyrus studied at 9–11 days in vitro), (2) the fusion protein used for immunization (TrpE–GluR3 246–458 versus GST–GluR3 246–455), (3) the method of removal of low molecular weight substances (repeated filtration through an Amicon Microcon filter versus Sephadex G50 chromatography), and (4) the product used for isolation of IgG (protein G sepharose from Pharmacia versus protein G agarose from Pierce). It is possible, for example, that our methods for removal of low molecular weight substances could have eliminated a light or heavy chain of the GluR3 antibody containing agonist properties. Likewise, if our cultures contained increased numbers of astrocytes available to buffer glutamate released from dying neurons, this could obscure detection of a GluR3 antibody with excitotoxic properties; nevertheless, this latter possibility would not be expected to obscure detection of a GluR3 antibody with agonist properties in the whole-cell recordings. Twyman et al. (1995) reported using filtrates or IgG isolated from serum; we have obtained equivalent results in both cytotoxicity and electrophysiologic assays using filtrates of plasma and serum. These various factors notwithstanding, we suspect that the specificity of the immune response mounted to the GluR3 antigen in the different rabbits is the most likely explanation for these divergent results. Consistent with heterogeneity of the immune response is the observation in this study that serum isolated from one of five GST–GluR3-immunized rabbits did not contain cytotoxic antibodies, despite exhibiting abundant anti-GluR3 IgG when titered using immunoblots (data not shown). Regardless of the factor(s) underlying the differences in electrophysiologic results, the absence of antibody-activated CNQX-sensitive currents, together with the insensitivity of the anti-GluR3-mediated injury to ionotropic glutamate receptor antagonists, argues against an excitotoxic mechanism of GluR3 IgG–mediated cytotoxicity in the present study.

Instead, our findings implicate a heretofore undescribed mechanism of glutamate receptor-dependent cell death. We propose that anti-GluR3 IgG binds GluR3 on rat cortical cells in vitro and triggers death by a complement-dependent mechanism. The assertion that this is IgG is based upon the selectivity of the Protein G affinity chromatography together with experiments demonstrating that the IgG fraction is necessary but not sufficient for the cytotoxicity. The assertion that this is GluR IgG rests upon the demonstration that the cytotoxic effects of the plasma are virtually abolished in fractions from which anti-GluR IgG has been virtually eliminated by affinity chromatography; by contrast, robust cytotoxic effects of plasma persist despite selective removal of the majority of anti-GST IgG. Whether affinity-purified anti-GluR IgG eluted from these columns combined with a source of complement could fully reconstitute the cytotoxic effects is uncertain; nevertheless, the virtual elimination of the cytotoxicity following selective removal of anti-GluR IgG implicates anti-GluR IgG as the predominant complement-activating IgG in this phenomenon. The assertion that this is anti-GluR3 IgG in particular rests upon the fact that immunocytochemical study of HEK cells transfected with single subunits of the closely related proteins GluR1, GluR3, and GluR6 disclosed immunoreactivity to GluR3- but not GluR1- or GluR6-transfected cells. The lack of correlation between anti-GluR antibody titers measured by immunoblot and either cytotoxic effects of antisera or disease in the present study is paralleled by similar findings in an autoimmune disease in which anti-receptor antibodies are pivotal in pathogenesis, namely myasthenia gravis. That is, the presence, but not the titers, of anti-acetylcholine receptor antibodies correlates with illness in both human myasthenia gravis and experimental autoimmune myasthenia gravis (Christadoss et al., 1985; Drachman, 1994). Whether this lack of correlation is due to the ability of subpopulations of anti-GluR3 to bind and/or activate complement is uncertain.

The assertion of a complement-dependent mechanism in the present study is based upon two observations. First, the activity of the rabbit plasma fraction required to reconstitute the cell-death effects of the IgG can be destroyed by heating to 56°C for 30 min, a treatment known to inactivate complement. Second, sCR1 inhibited the cell death–inducing effects of serum filtrate as well as the Protein G fraction required to reconstitute the cytotoxic effects of IgG. The similarity in the potency of inhibitory effects of sCR1 on lysis of sheep erythrocytes and the cytotoxic effects of rabbit sera on cortical cells strengthens the likelihood that the mechanism of the sCR1 effect involves inhibition of complement. Antibody-dependent destruction of cells through binding and activating complement has rich precedent in many nervous system diseases, including experimental allergic encephalomyelitis and potentially multiple sclerosis and Guillain-Barre syndrome (Morgan, 1993). Moreover, circumstantial evidence for a complement-dependent mechanism of cell injury by antibodies directed against a ligand-gated ion channel receptor has emerged from study of myasthenia gravis; immunocytochemical evidence of both IgG (Engel et al., 1979) and the MAC of complement (Engel and Arahata, 1987) at the neuromuscular junction suggests that anti-AChR antibodies damage the postsynaptic membrane at least in part through a complement-dependent mechanism. Given the predominant localization of GluR3 to neurons in the mixed cortical cultures studied here, it seems plausible that the anti-GluR3 IgG binds GluR3 on cortical neurons and activates the complement cascade; whether the cytotoxicity reflects lysis caused by MAC formation on these same neurons or an indirect mechanism involving other cell types is a topic for future study.

The presence of circulating antibodies to GluR3 in the serum of patients with active RE and the clinical improvement paralleling reduction of the antibody titers led us (Rogers et al., 1994; Andrews et al., 1996) to suggest that the antibodies themselves may damage the nervous system. For this suggestion to be correct, the antibodies must have deleterious properties and must gain access to the antigen. Since access of circulating IgG to antigens in the central nervous system is normally limited by the blood–brain barrier, we postulated that the blood–brain barrier must somehow be disrupted in RE to account for the response to plasma exchange. The present findings demonstrate that IgG has gained access to neuronal epitopes in the brain, in that abundant IgG-like immunoreactivity was evident

over a subset of neuronal cell bodies and their processes in deep layers of cortex in each of the two ill rabbits but not in any control rabbits or healthy GST–GluR3-immunized rabbits (all of which, nevertheless, had robust immune responses to GluR3). Increased IgG-like immunoreactivity was also evident in hippocampus but not in thalamus or cerebellum of the ill rabbits. The distribution of the IgG-like immunoreactivity revealed by immunocytochemistry is similar to that of GluR3 expression as detected by in situ hybridization of mature rat brain (Keinanen et al., 1990).

These immunohistochemical findings led to our demonstration of analogous findings in the brains of two of three patients with RE; that is, neurons—in particular, their cell bodies and apparent apical dendrites—exhibited IgG-like labeling in a pattern with striking similarity to that of the rabbits. Precisely how the IgG and members of the complement cascade gained access to the neurons is uncertain, but the paucity of detectable B cells in histochemical studies of brains of RE (Farrell et al., 1995) patients strengthens the likelihood that circulating IgG gained access through a defect in the blood–brain barrier, a suggestion consistent with the responsiveness of some of these patients to plasma exchange. We are attempting to test this idea by determining whether focal disruption of the blood–brain barrier in an asymptomatic animal with circulating cytotoxic anti-GluR3 will induce focal encephalitis and seizures.

The availability of anti-MAC antibodies useful for human study permitted detection of anti-MAC–like labeling of neuronal cell bodies and processes in the same regions of cortex demonstrating IgG-like labeling. The presence of anti-MAC–like labeling provides evidence for activation of the complement cascade, resulting in deposition of potentially cytolytic MAC on these neurons. Moreover, the fact that neurons in overlapping areas of adjacent or nearby sections were labeled by each antibody raises the possibility that the anti-neuronal IgG triggered activation of the complement cascade. The presence of MAC-like immunoreactivity on these neurons may provide an important clue to the pathogenesis of the neuronal degeneration and seizures of RE.

Experimental Procedures

Construction of Plasmid and Purification of Fusion Protein

GluR3 protein was expressed as a fusion protein linked to GST using the GST bacterial expression system (Pharmacia). A BamHI-SmaI restriction fragment encoding a portion of the N-terminal extracellular domain (residues 246–455) of rat GluR3 was subcloned into a *pGEX-KT* vector and used to transform E. coli. The GST protein was obtained by transforming bacteria with the *pGEX-KT* vector itself. The GST–GluR3 fusion protein was purified using SDS-PAGE and visualized by soaking the gel in 0.3 M $CuCl_2$ for 10 min. GST protein was purified either as described for GST–GluR3 or using glutathione-sepharose 4B according to the manufacturer's protocol; equivalent results were obtained with animals immunized with GST prepared by either method. Purified proteins were aliquoted and frozen at −80°C until use.

Antibody Production, Preparation of Plasma, and Purification of IgG

White New Zealand male rabbits weighing 2.5 kg were injected subcutaneously with 100 μg GST–GluR3 or GST in complete Freund's adjuvant, with subsequent injections in incomplete Freund's adjuvant. The first boost was given 2 weeks after the initial immunization, and the remaining boosts were given at 4 week intervals. Plasma was obtained by collecting blood in sodium heparin (1000 units/ml).

Sephadex G-50 chromatography was used to reduce the concentration of glutamate and other small molecular weight substances in plasma. The plasma was diluted 1:1 in Hank's Balanced Salt Solution (HBSS)/HEPES and passed over Sephadex G-50 (Pharmacia) columns twice; in initial experiments, this procedure removed >99% of free glutamate as demonstrated by monitoring ^{3}H-glutamate added to the plasma. IgG was purified with Protein G agarose (Pierce) affinity chromatography using 100 mM glycine-HCl (pH 2.5) for elution according to the manufacturer's instructions. The eluted immunoglobulin fraction was concentrated and equilibrated with HBSS/HEPES using centrifugal concentrators (Centricon 50, Amicon); the fraction exceeding 50 kDa was retained and stored at 4°C until use. In some experiments, the non-IgG portion of plasma was also collected, concentrated and equilibrated with HBSS/HEPES.

Protein concentrations were determined by modified Bradford protein assay (Coomassie Plus, Pierce). The plasma and portion of plasma not retained by Protein G agarose columns were filtered, aliquoted, and stored at −80°C.

Preabsorption and Immunoblot

Antibodies to GST and GST–GluR3 were removed from plasma by affinity chromatography with GST or GST–GluR3 bound to glutathione-sepharose 4B. GST or GST–GluR3 was mixed with glutathione-sepharose 4B at a concentration of 1 mg protein/ml resin. Plasma was incubated (plasma:resin, 2:1, vol/vol) with gentle agitation at 4°C for 16 hr and centrifuged, and the supernatant (either GST–GluR3 or GST-absorbed plasma) was used for subsequent experiments. To test the efficacy and specificity of preadsorption, TrpE–GluR3 (Rogers et al., 1994), GST–GluR3, GST, and rat-brain membranes were subjected to SDS-PAGE and transferred to Immobilon-P (Millipore) by electroblotting (100 V, 1 hr at 4°C). After fixation in methanol and acetic acid, the blot was blocked with 5% nonfat dry milk and 0.1% Tween 20 in 1× phosphate buffered saline (PBS) for 1 hr and then incubated for 1 hr in plasma diluted 1:1000 in blocking buffer. After washing for 15 min with three intermediate changes, blots were incubated with horseradish peroxidase-conjugated donkey anti-rabbit Ig (1:5000, Amersham) for 1 hr. Following several washes with 0.1% Tween 20 in 1× PBS, immunoreactivity was visualized with the enhanced chemiluminescence (ECL) detection system (Amersham). For detection of IgG in rabbit brain by immunoblotting, 30 μm cryostat sections of cortex (near those depicted in Figure 6B) were homogenized in 1× PBS, subjected to PAGE, transferred to Immobilon-P, and probed with goat anti-rabbit IgG (Jackson Lab, 1:10,000). For detection of IgM, the blot was stripped in 0.25 M glycine buffer pH 2.5 in 0.05% Tween 20 and reprobed with goat anti-rabbit IgM (Southern Biotech, 1:5000).

To detect the presence and relative amount of GluR3 antibodies, TrpE and TrpE–GluR3 were subjected to SDS-PAGE and transferred to Immobilon-P as described in the preceding paragraph, blocked with 5% dry milk, probed by rabbit sera (1:500 and 1:3000), and then incubated with goat-anti-rabbit IgG or IgM (Jackson Lab, 1:5000).

Tissue Culture

Mixed neuronal and glial cultures were prepared from embryonic day 18 rat cerebral cortices (Sprague-Dawley, Zivic Miller) as previously described (Patel et al., 1996). In brief, the cerebral cortices were dissected and enzymatically dissociated by incubation in Ca^{2+}- and Mg^{2+}-free HBSS supplemented with 10 mM HEPES and 0.25% trypsin for 20 min at 37°C. The tissue was rinsed and dispersed into single-cell suspension by gentle passage through a fire-polished Pasteur pipette. The cell suspension was centrifuged and resuspended in Minimum Essential Media (MEM) containing Earle's salts supplemented with 3 g/l glucose, 5% horse serum, and 5% fetal bovine serum (growth medium). The cells were plated at a density of 400,000–500,000 cells/well in poly-D-lysine–coated 24-well plates and maintained at 37°C in a humidified incubator with 5% CO_2/95% O_2 in growth medium. Medium was not replaced so as to reduce glial overgrowth and neuronal loss. Mature cells (2–3 weeks in vitro)

were used for all experiments. Dentate gyrus cells were prepared as described previously (Lerea et al., 1992). Poly-D-lysine coated coverslips were maintained for 9–11 days prior to electrophysiology experiments in a humidified incubator at 37°C in 95% O_2/5% CO_2.

For the assessment of cell death in cortical cultures, the growth medium was replaced with MEM and cells incubated with various treatments for 24 hr at 37°C. One hundred μM D-APV was routinely included to block NMDA receptor activation, except in the initial experiments in which the effect of APV was monitored. Receptor antagonists were added 15 min prior to AMPA or plasma filtrates. Neurotoxicity was determined by the measurement of LDH released into the supernatant media as previously described (Patel et al., 1996). LDH was measured by the method of Vassault (1983) using an ultraviolet automated plate reader (Molecular Devices). In some experiments, cell death was also monitored by uptake of trypan blue as previously described (Patel et al., 1996). To inactivate complement in the plasma or serum, samples were heated in a 56°C water bath for 30 min prior to incubation with cells. Alternately, cells were incubated with serum fractions in the presence of varying concentrations of sCR1 (Weisman et al., 1990).

Hemolytic Assay

To verify functionality of the sCR1, hemolytic assays were performed according to standard procedures (Kabat and Mayer, 1961). Briefly, sheep red blood cells suspended in Veronal-buffered saline (VBS [in mM]: 145 NaCl, 3.1 barbital, 1.8 Na barbital, 1 $MgCl_2$, and 150 μM $CaCl_2$) were coated with anti-Forssman's antibody (kindly provided by Dr. Michael Frank, Duke University) for 15 min at 30°C. After washing, coated red blood cells were exposed to rabbit serum either with or without various concentrations of sCR1 for 60 min at 37°C. Hemolysis reactions were stopped with the addition of ice-cold divalent-free VBS, the mixtures were centrifuged, and hemolysis was quantitated by measurement of the absorbance of the supernatant at 412 nm. Exclusion of either antibody or rabbit serum from the assay eliminated the hemolysis (data not shown).

Immunohistochemistry of Rabbit Brains

Rabbits were deeply anesthetized with pentobarbital (45 mg/kg i.p.) or ketamine/xylazine (50 mg/kg/10 mg/kg i.m.) and perfused transcardially with 500 ml of 0.9% NaCl followed by 1 liter of 4% paraformaldehyde in 0.1 M sodium phosphate buffer (1× PB [pH 7.4]) or 1 liter of 3% glutaraldehyde in 1× PBS at a rate of 75 ml/min. Blood (100 ml) was collected from each rabbit prior to saline perfusion. The brain was incubated in fixative overnight and cryoprotected in 30% sucrose in 1× PB. The brain was divided in the sagittal plane into two hemispheres. The right hemisphere was kept whole and frozen while the left hemisphere was divided into four coronal pieces, each of which was frozen in isopentane chilled in a dry ice/methanol bath. Thirty micrometer frozen sections were collected in 24-well culture plates containing 1× PBS and stored at 4°C until use.

All immunohistochemistry steps were carried out at room temperature. To reduce nonspecific binding of antibody, sections were blocked with 10% donkey serum in 1× PBS for 1 hr and incubated in secondary antibody (Jackson Immuno Research affinity-purified biotinylated donkey anti-rabbit IgG, 1:60000) in 1× PBS containing 2% BSA for 2 hr followed by three 10 min washes in 1× PBS and 2% BSA. Sections were treated with Vectastain ABC reagent (Vector Labs) and developed with 3,3′-diaminobenzidine tetrahydrochloride (DAB) as the chromogen. To reduce background, sections were preincubated in DAB alone for 5 min. Sections were mounted in 1× PBS on gelatin-coated slides, air dried, rinsed in water, dehydrated in ethanol, cleared in xylene, and coverslipped with Permount. Controls included sections from nonimmunized rabbits as well as sections not treated with secondary antibody.

Immunohistochemistry of Human Brain Samples

Surgically resected cortical tissue from three RE and three complex partial epilepsy patients was cut into blocks ~0.5–1.0 cm thick and immersion fixed at 4°C either for 72 hr in 2.5% paraformaldehyde/0.2% glutaraldehyde or for 1 week in 4% paraformaldehyde in 1× PB. Positive control tissue obtained at autopsy from a patient with AD was fixed for one month in 2.5% paraformaldehyde/0.2% glutaraldehyde at 4°C in 1× PB. Blocks were then cryopreserved in 20% sucrose in 1× PB and frozen in isopentane chilled in a dry ice/methanol bath. Thirty micrometer frozen sections were collected in 24-well culture plates containing 1× PBS and stored at 4°C until use. For the immunohistochemistry, sections from the RE, complex partial epilepsy, and AD cases were processed in parallel; all blocking, staining, and washing of sections was carried out in a 24-well tissue culture plate at room temperature, unless otherwise specified.

To block endogenous peroxidase activity, floating sections were incubated for 30 min in 0.3% H_2O_2 in 1× PBS. Following two washes in 1× PBS, sections were incubated for 1 hr in blocking buffer (1× PBS containing 0.1% Triton X-100, 2% bovine serum albumin, and 2% donkey serum) to reduce nonspecific background staining. During these treatments, the primary antibody (Jackson Immuno Research affinity-purified biotinylated anti-human IgG, 1:1000 or Calbiochem anti-MAC, 1:2000) was preadsorbed for 3 hr at room temperature in blocking buffer containing 20 mg/ml rat-brain protein prepared by acetone extraction (Harlow and Lane, 1988). The diluted primary antibody was subsequently spun briefly in a microfuge and the supernatant applied directly to the tissue sections. In some experiments, the antibody was incubated for an additional 3 hr at room temperature with 62 μg/ml of either the protein against which the antibody was raised (e.g., purified human IgG [Jackson Immunoresearch] or soluble MAC [Advanced Research Technologies]) or an irrelevant protein (e.g., purified rabbit IgG [Cappell] or C4 protein [Quidel], respectively). After the additional 3 hr incubation, the preincubated primary antibody mix was applied to the tissue sections. The sections were incubated for 1 hr at room temperature and then 36 hr at 4°C. After this, the sections incubated with anti-MAC were washed three times in blocking buffer and transferred to wells containing secondary antibody (Jackson Immuno Research affinity-purified biotinylated anti-rabbit IgG, diluted 1:400 in blocking buffer). One hour later, all sections were washed three times in 1× PBS containing 0.1% Triton X-100 and 2% bovine serum albumin; following this, the sections were incubated with Vectastain ABC reagent for 1 hr. Color development was allowed to proceed for 5 min in a substrate solution composed of 0.001% H_2O_2, 0.25 mg/ml DAB, and 6.25 mg/ml $NiSO_4$ in 0.175 M sodium acetate. Sections were mounted on gelatin-coated slides, air dried, dehydrated in graded alcohols, and coverslipped with Permount. Patterns of staining in these sections were ascertained by an investigator (J. O. M.) blinded to patient diagnosis, primary antibody, and preincubation protocol.

Immunocytochemistry of Transfected HEK Cells

Human embryonic kidney (HEK) 293 cells were transfected with expression plasmids containing either GluR1, GluR3, or GluR6 by the calcium phosphate method as described previously (Rogers, et al., 1994). Forty-eight hours after transfection, cells were fixed for 30 min at 4°C in PBS containing 4% paraformaldehyde, washed twice in 1× PBS, and incubated in blocking solution (PBS containing 2% horse serum, 0.5% BSA, 0.05% Tween-20, and 0.05% Triton X-100) for 1 hr followed by an overnight incubation at 4°C with primary antibodies diluted in blocking solution. The next day, cells were washed four times in blocking solution and incubated for 1 hr at room temperature with anti-rabbit IgG (1:200, Vector Labs) in blocking solution. Next they were washed four times in 1× PBS and incubated for 1 hr at room temperature with Vectastain Elite ABC reagent prepared according to the manufacturer's instructions (Vector Labs). The ABC reagent was removed with four washes in 1× PBS and the cells reacted with DAB solution prepared in 1× PBS to which 0.3% H_2O_2 had been added immediately before exposure to cells. After 3–5 min, cells were washed four times with PBS and maintained in PBS. Antisera used for positive controls for GluR1 and GluR6 were obtained from Dr. Scott Rogers as described by Rogers et al. (1994); antisera used as a positive control for GluR3 were raised against amino acids 373–395 of GluR3 as numbered by Keinanen et al. (1990) and affinity purified. Sera from GST- and GST–GluR3-immunized rabbits were used at a 1:1000 dilution.

Electrophysiology

Whole-cell recordings were obtained at room temperature from cells having a neuronal morphology with an Axoclamp-2A amplifier

equipped with a HS-2A x0.lL headstage, both from Axon Instruments. Cells were continuously perfused at 1 ml/min with a bath consisting of (in mM) 119 NaCl, 5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 30 glucose, and 25 HEPES (pH 7.4) equilibrated with 95% O_2/5% CO_2. For drug delivery, an array of blunt micropipettes ~20 μm in diameter was positioned about 100 μm from the cell. Drugs were ejected by 0.5–2.0 s pressure ejection from a Picospritzer II (General Valve). Recording electrodes having resistances of 2–4 MΩ were filled with a recording buffer of (in mM) 112 D-gluconic acid (2,3,4,5,6-pentahydroxy caproic acid), 17.5 CsCl, 49 HEPES, 2 EGTA, 3 $MgCl_2$, and 2 Na_2ATP (pH 7.2).

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