GluR3 Autoantibodies Destroy Neural Cells in a Complement-Dependent Manner Modulated by Complement Regulatory Proteins

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GluR3 autoantibodies have been implicated in the development of Rasmussen’s encephalitis, a rare neurodegenerative disease of humans characterized by epilepsy and degeneration of a single cerebral hemisphere. GluR3 autoantibodies are found in some Rasmussen’s encephalitis patients, and GluR3 antibodies raised in rabbits destroy cultured cortical cells in a complement-dependent manner. In this study, the cellular targets of anti-GluR3 antiserum-mediated cytotoxicity were examined in mixed primary neuronal–glial cultures of rat cortex. Unexpectedly, astrocytes were the principal target of the cytotoxic effects as assessed by immunohistochemistry and lactate dehydrogenase activity; neurons were destroyed to a lesser extent. Astrocyte vulnerability was rescued by transfection with complement regulatory proteins, and neuronal resistance was defeated by impairing complement regulatory protein function. Astrocyte death may occur in Rasmussen’s encephalitis, and destruction of this cell type may play a critical role in the progression of this disorder. The present findings suggest complement regulatory protein expression may in part determine the nature and severity of Rasmussen’s encephalitis and other complement-dependent nervous system diseases and thus underscore the need for a systematic investigation of the expression of all known complement regulatory proteins in healthy and diseased nervous system tissues.

Key words: GluR3 autoantibody; Rasmussen’s encephalitis; complement; complement regulatory protein; autoimmunity; epilepsy; glutamate receptor

Rasmussen’s encephalitis (RE) is an intractable epilepsy generally beginning during childhood or adolescence and characterized by progressive degeneration of a single cerebral hemisphere. Progressively worsening unilateral seizures and unihemispheric neurological problems, such as language deficits, hemiparesis, and hemisensory loss, develop in parallel with the hemispheric atrophy. Microscopic examination of resected brain tissue reveals focal inflammatory changes localized primarily to the cortex of the affected hemisphere, including microglial nodules, perivascular lymphocytic cuffing, meningeal infiltrates, neuronal loss, and gliosis (Rasmussen et al., 1958). Because the seizures are unresponsive to standard antiseizure pharmacotherapy, hemispherectomy is the standard treatment, typically leaving the child free of seizures but with a severe neurological deficit (Rasmussen et al., 1958; Oguni et al., 1991; Robitaille, 1991).

Although the etiology and pathogenesis of RE and the mechanism limiting the process to one hemisphere have yet to be completely elucidated, a series of recent studies has established autoantibodies and complement (C) as possible agents in disease progression. Immunization of rabbits with the glutamate receptor subunit GluR3 serendipitously led to an animal model recapitulating many of the principal features of RE and in turn to the discovery that many RE patients harbor antibodies directed at GluR3 (Rogers et al., 1994). Importantly, seizure severity and frequency are reduced in some RE patients treated with plasmapheresis or IgG-selective immunoadsorption, in parallel with reduction of GluR3 antibody titers (Rogers et al., 1994; Andrews et al., 1996; Antozzi et al., 1998). Additionally, some antibodies collected from immunized rabbits or humans with RE elicit excitatory currents in cultured neurons (Twyman et al., 1995). We have also found that rabbit anti-GluR3 can destroy primary mixed neuronal–glial cultures in a C-dependent manner (He et al., 1998). Additional support for IgG and C in RE pathogenesis emerged from immunohistochemical studies revealing IgG and C deposition on neurons in resected tissue from a subset of RE but not complex partial epilepsy patients (He et al., 1998; Whitney et al., 1999). These observations, along with the fact that most GluR3 in mixed neuronal–glial cultures are found on neurons (He et al., 1998), led to the hypothesis that anti-GluR3 binds selectively to neurons in culture, leading to antibody-dependent C activation in the vicinity of the neurons and selective neuronal destruction. The experiments described here reveal that anti-GluR3 unexpectedly does not selectively destroy neurons. Not only does anti-GluR3 destroy both neurons and astrocytes, neuronal death is more restricted and occurs more slowly than does astrocyte destruction. Neuronal resistance is reduced by impairing complement regulatory protein (CRP) function, and astrocyte resistance is improved by overexpression of CRPs.

MATERIALS AND METHODS

Unless otherwise indicated, all reagents were from Sigma (St. Louis, MO). Production of antisera and purification of IgG. White New Zealand male rabbits were immunized as described previously (Rogers et al., 1994; He et al., 1998). Briefly, rabbits weighing 2.5 kg were injected subcutaneously with 100 µg of glutathione S-transferase (GST)-GluR3, GST, trpE-GluR3, or trpE in complete Freund’s adjuvant. The GST-GluR3 fusion protein contains GluR3 residues 246–458 (Keinanen et al., 1990). Booster injections, given in incomplete Freund’s adjuvant, were given 2 weeks after the initial immunization and then at 4 week intervals. Serum was obtained by allowing blood to clot at 4°C followed by centrifugation. Sephadex G-50 chromatography was used to reduce the concentration of glutamate and other small molecular weight substances in serum. Serum diluted 1:1 in HBSS–HEPES (Life Technologies, Rockville, MD) was passed sequentially over two Sephadex G-50 columns (Amersham Pharmacia Biotech, Piscataway, NJ). Serum filtrates were filter-sterilized, aliquotted, and stored at −8°C. Because trpE-GluR3 and GST-GluR3 antisera are functionally indistinguishable in the experiments reported here, they will be collectively referred to as “anti-GluR3” or “GluR3 antisera.” TrpE and GST antisera will be referred to as “control antisera” for similar reasons.

Purified IgG was prepared with protein G agarose (Pierce, Rockford, IL) affinity chromatography according to the instructions of the manufac-
turer. The eluted immunoglobulin fraction was concentrated using Centricron centrifugal concentrators with a nominal molecular weight cutoff of 50 kDa (Millipore, Bedford, MA) and then dialyzed in four exchanges of PBS. Finally, the IgG fraction was further purified by affinity chromatography using a Protein A matrix (Pierce, Rockford, IL) and stored at 4°C in the presence of a cocktail of protease inhibitors.

**Tissue culture.** Mixed neuronal–glial cultures were prepared as described previously (He et al., 1998). Briefly, day 18 embryos were removed by cesarean section, the cerebral cortices were dissected, and the meninges were removed. Cortical pieces were minced into 1–2 mm2 pieces in HBSS (containing 1 mm HEPES; Life Technologies), minced, and then incubated with 0.25% trypsin in HBSS for 20 min at 37°C. Brain tissue was then rinsed twice in HBSS followed by two washes in plating medium (10% FBS in Neurobasal medium; Life Technologies) at room temperature (RT) for 10 min. In a separate tube containing Neurobasal medium, each plasmid to be transfected was added at a final concentration of 10 μg/ml and incubated at 37°C for 1 hr. In other experiments, cells were transfected using the calcium phosphate method as described previously (Sakurada et al., 1994; Quigg et al., 1995) (the first, at base 1145 encodes aspartate in place of valine; and the third, at base 1545, encodes leucine in place of asparagine). The construct was confirmed by DNA sequencing.

**Transfection.** Expression vectors containing green fluorescent protein (GFP), CD8, or mCherry were transfected using the Fugene 6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN) according to a method developed by VanDongen and colleagues (T. VanDongen [Duke University, Durham, NC], personal communication). Briefly, Fugene 6 was diluted 1:14 into Neurobasal medium (Life Technologies) containing 10 μg/ml DNase (Life Technologies). The tissue was then rinsed twice in HBSS, followed by two washes in plating medium (10% FBS in Neurobasal medium; Life Technologies) containing 0.001% H2O2, 0.25 mg/ml 3–3′ thymidine, 25 mg/ml glucose, 5 μg/ml bovine serum, and 5% horse serum; all from Life Technologies) containing 10 μg/ml DNase (Life Technologies). The tissue was then dispersed by trituration through a fire-polished Pasteur pipette. After centrifugation and resuspension, cells were plated at 8 × 10^5 cells/ml in 90-mm diameter petri dishes in plating medium. Cells were maintained at 37°C in 5% CO2–95% air in a humidified incubator until used for experiments on 14–16 days in vitro (DIV). The media was not exchanged during this time period. The numbers of cells differentiated were determined by a routine microscope.

**Cell毒性 assays.** On the day of the experiment, cultures were exposed to vehicle alone, equal concentrations of four control antisera (Fig. 1; Table 1, Experiment #1). One anti-GluR3 antiserum, which was not toxic at the concentrations tested here (0.5–1.0 mg/ml), and was excluded from this cell death analysis. In contrast, neither vehicle alone, equal concentrations of four control antisera (Fig. 1; Table 1, Experiment #1), nor commercial C′ (data not shown) produced significant destruction of cells when compared with untreated cultures. Importantly, the C′ inhibitor sCR1 reduced anti-GluR3-mediated death of astrocytes and neurons to 22 and <10%, respectively (Table 1, Experiment #2). sCR1 also prevented cell death as assessed by LDH assays (data not shown), confirming our previous report (He et al., 1998). Interestingly, the selective astrocyte L-amino adipate (t-AAA) (Khurpel et al., 1996) likewise destroyed not only most astrocytes (66%) but also a significant number of neurons (38%) (Table 1, Experiment #1). Our recent demonstration (He et al., 1998) that cytotoxicity is eliminated by selective removal of GluR3 antibodies from GST-GluR3 antisera, together with the present observations of equivalent cytotoxicity with anti-GluR3 raised against two distinct GluR3 fusion proteins, strongly implicate antibodies against GluR3 as the critical initiating factor.

**RESULTS**

**Cellular specificity of GluR3 antisera cytotoxicity.** To test the prediction that exposure of mixed neuronal–glial cultures to GluR3 antisera should selectively destroy neurons, GFAP-immunoreactive glia and Neu-N-immunopositive neurons were counted in cultures exposed for 24 hr to various antisera preparations. Unexpectedly, most astrocytes (85%) and significantly fewer neurons (47%) were destroyed by each of six GluR3 antisera raised against either the tripeptide GluR3 or GST-GluR3 fusion proteins (Fig. 1; Table 1, Experiment #1). One anti-GluR3 antiserum was not toxic at the concentrations tested here (0.5–1.0 mg/ml) and was excluded from this cell death analysis. In contrast, neither vehicle alone, equal concentrations of four control antisera (Fig. 1; Table 1, Experiment #1), nor commercial C′ (data not shown) produced significant destruction of cells when compared with untreated cultures. Importantly, the C′ inhibitor sCR1 reduced anti-GluR3-mediated death of astrocytes and neurons to 22 and <10%, respectively (Table 1, Experiment #2).
Table 1. Summary of cell count statistical comparisons performed

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Condition 1</th>
<th>Condition 2</th>
<th>p value</th>
<th>Condition 3</th>
<th>p value</th>
<th>Condition 4</th>
<th>p value</th>
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<tr>
<td>% Astrocyte death after 24 hr</td>
<td>Vehicle</td>
<td>Anti-GluR3</td>
<td>&lt;0.001</td>
<td>Control sera</td>
<td>1-AA (*)</td>
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<tr>
<td>% Neuron death after 24 hr</td>
<td>Vehicle</td>
<td>Anti-GluR3</td>
<td>&lt;0.001</td>
<td>Control sera</td>
<td>1-AA (*)</td>
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<tr>
<td>% Cell death after GluR3 antisera for 24 hr</td>
<td>Astrocyte</td>
<td>Neuron</td>
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<td><strong>Experiment #2</strong></td>
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<tr>
<td>% Astrocyte death after GluR3 antisera for 24 hr</td>
<td>Without sCR1</td>
<td>With sCR1 (*)</td>
<td>&lt;0.01</td>
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<tr>
<td>% Neuron death after GluR3 antisera for 24 hr</td>
<td>Without sCR1</td>
<td>With sCR1 (*)</td>
<td>&lt;0.01</td>
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<tr>
<td>% Propidium iodide + astrocytes after 4 hr</td>
<td>Vehicle</td>
<td>Anti-GluR3</td>
<td>&lt;0.01</td>
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<tr>
<td>% Propidium iodide + neurons after 4 hr</td>
<td>Vehicle</td>
<td>Anti-GluR3</td>
<td>&lt;0.01</td>
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<tr>
<td>% Propidium iodide + cells after anti-GluR3 for 4 hr</td>
<td>Astrocytes</td>
<td>Neurons</td>
<td></td>
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<td><strong>Experiment #4</strong></td>
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<tr>
<td>% Cells labeled by anti-GluR3</td>
<td>&lt;1</td>
<td>66</td>
<td>&lt;0.001</td>
<td>Control sera</td>
<td>Anti-GluR3</td>
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<tr>
<td>% Neurons labeled by antisera</td>
<td>7</td>
<td>66</td>
<td>&lt;0.001</td>
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<td><strong>Experiment #5</strong></td>
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<tr>
<td>% Cells labeled by anti-CD59</td>
<td>4</td>
<td>97</td>
<td>&lt;0.0001</td>
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<tr>
<td>% Propidium iodide + neurons after anti-GluR3 for 4 hr</td>
<td>Vehicle treat</td>
<td>PI-PLC treat</td>
<td>CD59 treat</td>
<td></td>
<td></td>
<td>MOPC treat</td>
<td>NS</td>
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<tr>
<td>% Propidium iodide + astrocytes after anti-GluR3 for 4 hr</td>
<td>Vehicle treat</td>
<td>PI-PLC treat</td>
<td>CD59 treat</td>
<td></td>
<td></td>
<td>MOPC treat</td>
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<tr>
<td>% Astrocyte death after anti-GluR3 for 4 hr</td>
<td>CD8 transfection</td>
<td>CD59 transfection</td>
<td>Crry transfection</td>
<td>&lt;0.05</td>
<td>8</td>
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<tr>
<td>% Astrocyte death after 1-AA for 4 hr</td>
<td>CD8 transfection</td>
<td>CD59 transfection</td>
<td>Crry transfection</td>
<td>&lt;0.05</td>
<td>8</td>
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<tr>
<td>% Astrocyte death after anti-GluR3 for 4 hr</td>
<td>CD59 transfection</td>
<td>Crry transfection</td>
<td>Crry transfection</td>
<td>&lt;0.05</td>
<td>8</td>
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</table>

Cell count data was collected for seven of the sets of experiments reported in this paper. Within each experiment, one or more comparisons were made between the various experimental conditions tested in each experiment. Each line represents one set of comparisons between the cell count data for each of the experimental conditions (Conditions 1–4, labeled individually for each entry), and the p values, derived from Student’s t tests or Tukey t tests performed post hoc to one- or two-way ANOVA, reflect the results of statistical comparisons among the conditions. For all but the last three lines, all cells in three random fields per well were counted, commonly amounting to ~200 cells per field in the control conditions (and fewer in the toxin-treated wells). For the experiments summarized in the last three lines, all GFP-transfected cells were counted in each well. Typically, at least three wells were tested per condition per experiment. Each experiment was performed at least three times, except where indicated with an asterisk, when the experiment was performed twice with equivalent results. The data represent pooled results from replicate experiments.

Table 2. Summary of LDH assay statistical comparisons performed

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Condition 1</th>
<th>Condition 2</th>
<th>p value</th>
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<tbody>
<tr>
<td><strong>Experiment #6</strong></td>
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<tr>
<td>LDH release (% vehicle) after anti-GluR3 antisera for 24 hr</td>
<td>Vehicle pretreat</td>
<td>PI-PLC pretreat</td>
<td>&lt;0.001</td>
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<tr>
<td><strong>Experiment #8</strong></td>
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<tr>
<td>LDH release (% vehicle) after anti-GluR3 antisera for 24 hr</td>
<td>Vehicle cotreat</td>
<td>CD59 cotreat</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td><strong>Experiment #9</strong></td>
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<tr>
<td>LDH release (% vehicle) after KA for 24 hr</td>
<td>Vehicle</td>
<td>Anti-GluR3 IgG</td>
<td>NS</td>
</tr>
<tr>
<td>LDH release (% vehicle) in presence of C6d serum + C6</td>
<td>100</td>
<td>132</td>
<td>&lt;0.05</td>
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</tbody>
</table>

Each line presents the results of comparisons between Conditions 1 and 2; p values are from Tukey t tests performed post hoc to two-way ANOVA run for each experiment. Each number represents enzymatic activity units present in culture supernatants, expressed as percent LDH values from vehicle-treated control wells, and is derived from at least three experiments in which three wells tested per condition were tested.

To begin to investigate the unexpected preferential destruction of astrocytes, cells were examined after 4 hr exposure to anti-GluR3 or other serum samples. In preliminary work, cell death as assessed by analysis of LDH release was first detected at 4 hr, increasing in magnitude during the subsequent 20 hr. Cultures were incubated with propidium iodide for 30 min before fixation to label dead or dying cells. In these experiments, astrocytes were labeled with anti-GFAP and neurons were stained for MAP-2 to facilitate qualitative evaluation of modest morphological changes. Although neurons and astrocytes both appeared normal in cultures exposed to vehicle alone, scattered confluent patches of astrocytes in cultures exposed to anti-GluR3 at 1 mg/ml were swollen or...
propidium iodide after treatment with antisera. In contrast, iodide uptake in 12% of astrocytes, 47% of astrocytes had taken up arrows
pidium iodide-negative at this time point (Fig. 2, the great majority of neurons were healthy-appearing and pro-
tional numbers of both glia and neurons. *

morphological changes seen in damaged (red arrows) astrocytes. In contrast to the astrocytes, the great majority of neurons were healthy-appearing and pro-
pidium iodide-negative at this time point (Fig. 2, right panel, green arrows). Blinded cell counts (Table 1, Experiment #3) revealed that, whereas exposure to vehicle alone resulted in propidium iodide uptake in 12% of astrocytes, 47% of astrocytes had taken up propidium iodide after treatment with antisera. In contrast, <7% of neurons were stained by propidium iodide, irrespective of treatment.

One possible explanation for selective destruction of astrocytes at early time points by anti-GluR3 is that large numbers of anti-
bodies might bind to these cells and fix C'. To explore antibody binding to glia, unfixed neuronal–glial cultures were incubated with various antisera or purified IgG for 30 min and then counterstained for GFAP or MAP-2. The percent of GFAP- or MAP-2-positive cells labeled by the various antisera was then determined by blinded cell counting. Whereas no more than 3% of astrocytes were labeled by any antisera or antibody tested, on average 66% of neurons were labeled on their somata and processes by the cyto-
toxic GluR3 antisera (Fig. 3; Table 1, Experiment #4). This staining pattern was observed with six different cytotoxic antisera and purified IgG analyzed in multiple experiments and is consistent with the predominantly neuronal expression of GluR3 in mixed neuronal–glial cultures (Eshar et al., 1993; He et al., 1998). Only 7% of neurons were labeled in cultures exposed to three control antisera (Table 1, Experiment #4), two commercial rabbit serum sources, or the single anti-GluR3 serum that was not cyto-
toxic at the concentrations used here. This last antisera also failed to stain GluR3-transfected HEK cells, whereas the other GluR3 antisera stained GluR3 but not mock-transfected HEK cells (data not shown). Thus, only cytotoxic antisera contain anti-
GluR3 antibodies capable of binding the antigen in a living cell, and when exposed to mixed neuronal–glial cultures, these antibod-
ies preferentially bind neurons.

**Cellular specificity of C' regulatory protein expression**

That neurons abundantly bind the IgG in GluR3 antisera predicts they would experience the greatest C’ attack. Yet neurons are not the cells most rapidly killed by GluR3 antisera. Instead, astrocytes, which apparently bind little high-affinity IgG in the GluR3 antisera, are rapidly destroyed by anti-GluR3. We hypothesized that neu-
rons may resist the toxic effects of anti-GluR3 better than astro-
cytes because they express higher levels of CRPs than do astro-
cytes. CRPs are a family of proteins that protect self tissues from attack by inhibiting C’ activation and deposition (for review, see Ashgar, 1998). The fact that dying astrocytes appeared to be swollen suggested that CRP deficiencies had allowed membrane insertion of the cytotoxic attack complex (MAC). The MAC is a multimeric 100 Å pore composed of the C’ factors C5b, C6, C7, C8, and multiple copies of C9, which allows ions and water free passage across the membrane of an attacked cell, causing cell swelling and lysis (Austyn and Wood, 1993). The CRP CD59 prevents MAC formation by preventing incorporation of C9 into the developing MAC (Morgan and Meri, 1994). To explore whether neurons express higher levels of CD59 than do astrocytes, cultures were stained with an anti-CD59 antibody (Fig. 4; Table 1, Experiment #5). CD59 was found on virtually all (97%) GAP-43-
positive axonal processes. In contrast, only 4% of GFAP-positive astrocytes expressed CD59. Importantly, substantial immunoreac-
tivity was also found in these experiments on subsets of MAP-2-
positive neuronal dendrites and neuronal somata (data not shown). Thus, whereas astrocytes express little CD59, neurons are coated from axon to dendrite with this protective protein.

**CRP inhibition, MAC formation, and cell death**

The fact that CD59 is anchored to the cell membrane through a glycosylphosphatidylinositol (GPI) linker can be exploited to test whether neuronal anti-GluR3 resistance is attributable to high CD59 expression levels. Cultures were treated with PI-PLC to remove GPI-anchored proteins such as CD59. As measured by LDH assay, PI-PLC-pretreated cultures were far more sensitive to low concentrations of anti-GluR3 than were vehicle pretreated cultures (Fig. 5, black vs white bars; Table 2, Experiment #6). The effects of PI-PLC were specific in that there was no significant effect on the toxicity produced by the neurotoxin KA or the astrotoxin 1-AA. Moreover, basal cell viability, assessed in vehicle-challenged cultures, was unaffected by PI-PLC pretreatment. Heat-inactivated PI-PLC had no effect on the toxicity produced by any agent.

To determine whether PI-PLC pretreatment sensitizes otherwise resistant neurons to anti-GluR3, a set of immunocytochemistry experiments was performed in which vehicle- and PI-PLC-
pretreated cultures were challenged for 4 hr with vehicle or anti-
GluR3. Dying cells were labeled by incubation with propidium iodide during the last 30 min. The percent of GFAP- and MAP-2-positive cells also positive for propidium iodide was then deter-
mined by blinded cell counting. Whereas <7% of neurons in cultures pretreated with vehicle and challenged with anti-GluR3 were propidium iodide-positive, 72% of neurons in PI-PLC-
pretreated cultures challenged with anti-GluR3 were propidium iodide-positive, bereft of neurites, and swollen (Table 1, Experiment #7). Images representative of three experiments are shown in Figure 6 (top row). Astrocytes were also sensitized by PI-PLC pretreatment, with the percentage of astrocytes labeled by propidium iodide after 4 hr incubation with anti-GluR3 rising from ~50% in the vehicle-pretreated cultures to >80% in the PI-PLC-
pretreated cultures (Table 1, Experiment #7). No significant changes were observed in the numbers of propidium iodide-labeled astrocytes or neurons, or in their morphology, in vehicle-
challenged wells, irrespective of pretreatment.

CD59 is just one of multiple proteins removed by PI-PLC. To directly test the role of CD59, a neutralizing anti-CD59 monoclonal antibody was used. Cultures were treated with GluR3 antisera in

![Figure 1](https://example.com/f1.png)

**Figure 1.** Anti-GluR3 destroys astrocytes and neurons after 24 hr exposure. Mixed cultures were incubated with vehicle, various serum samples, or 1- AA for 24 hr, fixed, and then immunostained for GFAP (white bars) or NeuN (black bars) to label astrocytes or neuronal nuclei, respectively. Data presented are averaged cell counts from three random fields per well from several experiments. Whereas anti-GluR3 (n = 6 antisera) destroy the majority of astrocytes and approximately half the neurons, control antisera (n = 5) destroy <10% of cells. The astrotoxin 1-AA also destroys substantial numbers of both glia and neurons. **p < 0.05 compared with control groups; **p < 0.001 compared with vehicle or control serum-treated groups.**
the presence of 100 μg/ml of this neutralizing antibody (clone 6D1) or a nonspecific control antibody (clone MOPC-31C). Note that neither of these antibodies can itself fix C₉, being of the IgG1 isotype (Hughes et al., 1992; Rushmere et al., 1997). Whereas only 7% of neurons were propidium iodide-positive in cultures challenged with GluR3 antisera alone, 37% were positive in cultures challenged with GluR3 antisera in the presence of anti-CD59 (Table 1, Experiment #7). In contrast, only 11% of neurons were stained by propidium iodide in cultures challenged with GluR3 antisera in the presence of the MOPC control antibody. Images representative of four experiments are shown in the bottom row of Figure 6. Astrocyte death after challenge with anti-GluR3 was not appreciably affected by cotreatment with anti-CD59 (or anti-MOPC) (Table 1, Experiment #7), presumably reflecting the fact that these cells express little CD59. Furthermore, no significant changes in the numbers of propidium iodide-positive neurons or astrocytes were noted in cultures challenged with vehicle in either cotreatment group. As an independent indicator of cell death, LDH release was measured in cultures subjected to these cotreatment regimens. LDH release in anti-CD59, GluR3 antiserum cotreated cultures was 132% of that from cultures treated with anti-GluR3 alone (218 vs 165% of baseline control, respectively); cotreatment with anti-MOPC had no significant effect on cell death. Notably, KA toxicity was not affected by either antibody (LDH values ≈230% of baseline in each KA group) (Table 2, Experiment #8).

Together, these results indicate that GPI-anchored proteins such as CD59 play an important role in neuronal C₉ defense. The greater neuronal death in GluR3-challenged cultures after PI-PLC treatment than anti-CD59 treatment (79 vs 37%) may reflect the fact that PI-PLC removes all GPI-anchored proteins, including other CRPs that may provide additional C₉ defense.
enhanced astrocyte death after PI-PLC treatment may reflect removal of additional GPI-anchored CRPs from the membrane of this cell type.

Although the results of the CD59 inhibition experiments imply MAC-mediated swelling and lysis as the mechanism of C'-mediated death, we could not directly assess MAC deposition on astrocytes (or on neurons with debilitated CD59) because the majority of commercially available anti-C' antibodies are neither specific for rabbit C' nor sufficiently robust to enable useful immunocytochemical analyses. Moreover, C' cytotoxicity can in princi-
These peptides, C3a and C5a, potently activate cells bearing specific receptors such as T-cells, macrophages, neurons, astrocytes, and microglia (Armstrong et al., 1990; Yao et al., 1990; Austyn and Wood, 1993; Nolte et al., 1996; Nataf et al., 1999). Therefore, to determine by a functional endpoint whether anti-GluR3-mediated cell death results specifically from MAC deposition as opposed to downstream factors C3 and C5. This bystander mechanism has been established in C6-deficient serum and C5b-7 formation on astrocytes suggests they would permit more construction of the full MAC channel.

CRP overexpression, cytoprotection, and the bystander hypothesis

The findings presented so far suggest that neuronal resistance results from high-level CRP expression and that astrocytes are lysed by MACs inserted into their membranes. However, although the C6d experiments indicate that MAC formation is essential for cell death, they do not prove MAC formation must occur specifically on astrocytes for astrocytes to die. For instance, MAC deposition on neurons may trigger the release of a compound that then kills astrocytes. One way to distinguish these possibilities is to examine the ability of transiently overexpressed CD59 to protect astrocytes against anti-GluR3-mediated destruction, because the role of CD59 is to prevent MAC formation. To address this question, cultures were cotransfected with GFP and CD59 and then challenged with anti-GluR3; the number of surviving GFP-positive astrocytes were compared with cultures co-transfected with GFP and CD8 as a control. Whereas anti-GluR3 destroyed ~40% of CD8-cotransfected astrocytes (Fig. 8, hatched vs white bar in left group of bars), anti-GluR3 destroyed <8% of CD59-cotransfected astrocytes (Fig. 8, middle set of bars; Table 1, Experiment #10). CD59 specifically protects against C’ attack because t-AAA destroyed >80% of astrocytes in both transfection conditions (black bars) (Table 1, Experiment #10). When combined with the C6d work, these observations indicate that MAC formation on astrocytes is required for astrocyte destruction.

If cell death is initiated by anti-GluR3 binding to GluR3 protein on neurons, with consequent C’ activation on this same cell type, how can MAC-dependent astrocyte death occur? One possibility is that C’ originally activated on neurons envelops and ultimately kills neighboring astrocytes. According to this “bystander” process, as the C’ cascade activates on neurons, a transiently water-soluble, incomplete MAC (C5b-7) deposits on both neurons and bystander astrocytes. To the extent a cell expresses CD59, construction of the complete MAC will be inhibited. The relative lack of CD59 on astrocytes suggests they would permit more construction of the full MAC than neurons and therefore suffer more C’-mediated lysis. This bystander mechanism has been established in C’-mediated destruction of other closely juxtaposed cell types (Park et al., 1997). An alternative possibility is that antibodies binding to neurons have no effect on astrocyte death at all; instead, small numbers of antibodies may bind to astrocytes and fix C’ directly on them. To distinguish these possibilities, the protective effect of overexpressing a CRP that acts at earlier stages of the C’ cascade was examined. Crry was chosen because (1) it is a multifunctional CRP that potently inhibits cascade activation and (2) immunocytochemistry experiments revealed Crry is selectively expressed on neuronal processes and not astrocytes, an expression pattern exactly matching that of endogenous CD59 (Fig. 4). If astrocytes are destroyed by the insertion of C5b-7, then Crry overexpression should fail to protect because this CRP acts upstream of C5b-7 formation. If the bystander process is not involved, however, and C’ activation is
initiated directly on astrocytes, then astrocytes expressing Crry should be protected because the protein will halt the cascade as soon as it is activated. Only 8% of Crry-transfected astrocytes were destroyed by anti-GluR3, a level of protection equivalent to that afforded by CD59 transfection (Fig. 8, right set of bars; Table 1, Experiment #10). Importantly, t-AA destroyed >80% of astrocytes in this group as well, confirming the C9-specificity of Crry protection (Table 1, Experiment #10). That transfection of either CD59 or Crry protects astrocytes against the toxic effects of GluR3 antibodies binds rapidly to neurons and not astrocytes, implying that both early and late C9 cascade components are deposited on astrocytes and that bystander processes do not appear to contribute significantly to astroglial death.

DISCUSSION

Overview

This study investigates the cytotoxic actions of anti-GluR3 in primary cultures containing a mixture of astrocytes and neurons. The principal findings are fivefold. (1) Whereas the majority of GluR3 antibodies binds rapidly to neurons and not astrocytes, anti-GluR3 antiserum preferentially and unexpectedly destroy astrocytes. Prominent neuronal damage is only seen at later time points, and even after 24 hr, the proportion of astrocytes destroyed exceeds that of neurons. (2) GluR3 antiserum-mediated cell death requires complement (C) activation because cytotoxicity can be inhibited by sCR1. (3) The C6-deficient serum experiments reveal that anti-GluR3 cytotoxicity specifically requires construction of the MAC. (4) Neuronal resistance to C9 attack is enabled by high levels of CRPs because resistant neurons can be sensitized by interfering with CD59 function. (5) Conversely, astrocytes can be protected from C9 attack by transfection with either of two CRPs, CD59, or Crry. Together, these observations suggest a model explaining how GluR3 antiserum damage both neurons and astrocytes (Fig. 9). Succinctly, anti-GluR3 binding to both neurons and astrocytes activates C9 on both cell types; that most antibodies bind to neurons suggests that extensive C9 deposition occurs on this cell type. However, high CRP expression levels by neurons protect them from C9-mediated destruction, whereas low astrocytic CRP expression leaves this cell type incapable of preventing MAC construction. Consequently, astrocytes and not neurons suffer lytic death at early time points.

There are several plausible explanations for the neuronal death that occurs at later time points. One is that neurons die principally as a consequence of previous destruction of astrocytes by anti-GluR3, as suggested by the similar pattern of neuronal and glial damage caused in the present work by the t-AA, a selective astrotoxin (Khurgel et al., 1996). Potential mechanisms of neuronal death include loss of astrocyte-derived BDNF or other growth factors upon which neurons are known to depend for survival in primary culture (Banker, 1980; Barde, 1989; Eddleston and Mucke, 1993) or from toxic accumulations of, for example, extracellular glutamate normally buffered by astrocytes (Choi, 1992; Rothstein et al., 1996; Tanaka et al., 1997). Alternatively, neurons may die only after exhausting their ability to resist C9 attack. For instance, CD59 complexes stoichiometrically with membrane-deposited C8 and C9 in preventing MAC formation, and because the CD59 stock of a cell is finite, it may eventually be depleted (Rollins et al., 1991; Morgan and Meri, 1994).

The bystander hypothesis, CRP expression levels, and neurological disease

The experiments with sCR1 and C6-deficient serum indicate that astrocyte destruction requires not only activation of the C9 cascade but also construction of the MAC channel. However, these experiments do not prove that cytotoxic MAC deposition occurs directly on astrocytes; conceivably, neuronal MAC deposition triggers release of a compound that then kills astrocytes. The CD59 transfection experiments were undertaken to investigate this possibility and revealed that astrocytes are protected by CD59 expression, implying that MAC deposition does occur directly on astrocytes. These findings beg the additional interesting question of whether astrocyte MAC deposition could result from C9 activation occurring in the vicinity of GluR3 on neurons. The bystander hypothesis predicts that MAC deposition on astrocytes occurs when incomplete MACs spread from neurons to nearby defenseless astrocytes. This possibility was tested by transfection with Crry, which will not protect astrocytes if the bystander hypothesis is correct because Crry acts at a stage earlier than formation of the free-floating nascent MAC. That expression of Crry on astrocytes enhances astrocyte resistance implies that early C9 components are deposited directly on astrocytes.

Together, these transfection experiments indicate that the C9 cascade is initiated and completed directly on astrocytes and that the bystander hypothesis is incorrect. Because of the paucity of astrocyte CRPs and the catalytic manner in which C9 cascade activation proceeds, even a small number of antibodies binding to astrocytes may trigger sufficient C9 activation to kill these cells. The antibodies may be so few in number or of such low affinity that they remain immunocytochemically undetectable. Based on previous
immunodepletion experiments in which removal of GluR3 but not GST antibodies from GST-GluR3 antisera completely abrogated toxicity, the astrocyte-binding antibodies are likely directed against GluR3 (He et al., 1998). Astrocytes express multiple types of glutamate receptors in vitro, albeit at lower levels than neurons, including AMPA receptors containing GluR3 (for review, see Steinhauser and Gallo, 1996).

Astrocytes express GluR3 in vivo and may likewise suffer antibody-mediated C-dependent destruction in RE given the low CRP expression levels described by immunocytochemistry and in situ hybridization studies in the literature (Meri et al., 1991; Johnstone et al., 1993; Funabashi et al., 1994; Vedeler et al., 1994; Zajicek et al., 1995; Koski et al., 1996; Davoust et al., 1999; Spiller et al., 1989). However, the CRP family is a large one, and a comprehensive investigation of the cellular (i.e., neuronal vs glial) localization of all known family members in diseased and normal nervous system tissues from humans and the rodents used for neurological disease models remains to be completed. The pivotal role cellular CRP expression plays in vitro in protecting against C-mediated attack underscores the need for such a systematic description. Indeed, deficient CRP expression or expression of a defective isoform of a CRP in vivo may constitute an important risk factor for RE, myasthenia gravis, multiple sclerosis, Alzheimer’s disease, and other neurological disorders in which autoantibodies and C have been implicated (McRae et al., 1996; Whitney and McNamara, 1999). Although the ideas of CRP function and dysfunction are a central concept in the organ transplantation field (McNamara, 1999). Although the ideas of CRP function and dysfunction are a central concept in the organ transplantation field (McNamara, 1999). Although the ideas of CRP function and dysfunction are a central concept in the organ transplantation field (McNamara, 1999).}

Astrocyte death and RE progression

Whether or not RE patients suffer from defective CRP expression, this disease is classically defined by broad neuronal death and reactive astroglia. However, the drastic hemispheric degeneration common in advanced RE cases in which large parts of a hemisphere are destroyed is not likely attributable to the exclusive loss of neurons, because glia are the predominant cell type and probably are a major contributor to tissue volume in the CNS. Additionally, the magnitude of astrocyte loss may be obscured by the proliferation of normal glia responding to tissue damage. Damage to astrocytes in the context of neurodegeneration and reactive astroglia has been observed in other pathological conditions. For instance, degenerating astroglia have been detected in the brains of patients with frontotemporal dementia, a disease characterized by widespread layer-specific neuron loss and reactive astroglia (Martin et al., 1998). In this study, degenerating astrocytes were found scattered among proliferating reactive astroglia with the overall number of intact astrocytes being similar to control specimens.

How might such astrocyte death contribute to the neuronal loss, seizures, gliosis, and CNS inflammation typical of RE? Perhaps most simply, as alluded to above, neurons may die for lack of astrocyte-derived trophic support or from exposure to toxic compounds no longer sequestered by astrocytes. Also, seizures may result from deficient glutamate buffering (Rothstein et al., 1996; Tanaka et al., 1997). Additionally, cell death will activate neighboring astrocytes and microglia, both of which rapidly respond to even minute changes in the CNS environment by assuming an activated posture that includes proliferation, upregulation of numerous cell-surface receptors, and other genes, and for microglia the additional capacity to phagocytose cellular debris (Eddleston and Mucke, 1993; Kreutzberg, 1996; Streit, 1996). Fcy, CR1, and CR3, which bind to IgG, C3b, and C4b, respectively, are among the upregulated microglial receptors, and interactions between these receptors and their membrane-bound ligands may trigger phagocytosis (Austyn and Wood, 1993). Brain sections from RE patients often contain neurons surrounded by microglia thought to be engaged in neuronophagia (Robitaille, 1991). We speculate that, after proliferating and clearing away cellular debris, the glia remain as the microglial nodules and glotic scars often noted in RE specimens (Robitaille, 1991). Finally, high local concentrations of C5a may also underlie the formation of perivascular lymphocytic cuffs, because T-cells bear C5a receptors that mediate chemotaxis up C5a gradients in vitro (Nataf et al., 1999).

A recent study of the role reactive astrocytes play in wound repair in the CNS provides evidence in support of some of these suggestions (Bush et al., 1999). Transgenic mice were generated whose astrocytes expressed the viral thymidine kinase gene driven by GFAP promoter. Reactive astrocytes were selectively destroyed after a recent experimental stab wound with systemically administered ganciclovir because thymidine kinase levels rose in parallel with GFAP transcription around the wound site. Reactive astrocyte ablation was associated with exacerbated inflammation at the wound site, defective repair of the blood–brain barrier (BBB) as judged by prolonged permeability to circulating proteins, and destruction of neighboring neurons in a glutamate receptor activation-dependent manner. Extrapolating these findings to RE, astrocyte destruction may enable inflammatory cells, pathogenic IgG, and C easy access to the neural environment through a persistently damaged BBB. The phenomena that we postulate stem from these agents (inflammation, seizures, and cell death) would represent not only an exacerbation of the disease but also a means of disease perpetuation, because activated inflammatory cells, seizures, and C activation products can disrupt the BBB (Cornford and Oldendorf, 1986; Hollerhughe et al., 1989; Kadomugamuwa et al., 1989; Steiner, 1996). Consistent with the notion that peripheral pathogenic factors enter the CNS via a disrupted BBB are the immunocytochemical findings of preferential perivascular IgG and C deposition in brain samples from a subset of RE patients (Whitney et al., 1999) and responsiveness of some RE patients to plasma exchange or removal of circulating IgG (Rogers et al., 1994; Andrews et al., 1996; Antozzi et al., 1998). Examined in the context of these studies, C-mediated astrocyte damage may be a pivotal event in RE pathogenesis that ensures the development of a chronic disease marked by seizures, neuronal and glial death, inflammation, and gliosis.

REFERENCES


