Circulating Autoantibodies Recognize and Bind Dying Neurons Following Injury to the Brain

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Abstract. While it is known that autoimmune cells can protect against cell damage following traumatic injury of the brain, the role of autoantibodies in brain injury is less clear. Here we present evidence in adult rats that following a cortical lesion of the brain, circulating IgG autoantibodies bind to dying neurons in the vicinity of the lesion. At intervals that ranged from 4 h to 7 days after making a unilateral lesion of visual cortex, we observed neurons near the lesion that were immunopositive for rat IgG. Many of these IgG-positive neurons were in advanced stages of degeneration. The magnitude of the immunostaining observed was directly proportional to the percent reactivity to rat IgG of the antibodies that were used. Preadsorption of the antibodies with rat serum eliminated the immunostaining. In addition, immunostaining for serum albumin in sections through the cortical lesion was negative, supporting the conclusion that the positive staining for IgG does not result from the passive diffusion of serum proteins into injured cells. Instead, the evidence presented here strongly suggests that naturally occurring IgG autoantibodies bind specifically to dying neurons in the injured brain. We propose that this autoantibody binding may participate in the phagocytosis and removal of injured neurons.

Key Words: Autoimmunity; Blood-brain barrier; Cortical lesion; Immunoglobulins; Neural cell death.

INTRODUCTION

For many years, autoimmunity has been associated solely with disease. For example, enteric infection with Campylobacter jejuni can lead to the production of anti-ganglioside antibodies that cause demyelination or Guillain-Barré syndrome (1). Such molecular mimicry mechanisms, which involve a foreign antigen mimicking a self antigen and can lead to an aberrant autoimmune response, also are thought to be involved in multiple sclerosis (2, 3) and Stiff-Man syndrome, a disease characterized by rigidity and spasms (4).

However, a low level of autoimmunity is necessary for normal function (5). For instance, the maturation and survival of lymphocytes requires autoantigen (6). Furthermore, substantial evidence suggests an important and complex role for autoimmunity in lesions of the central nervous system (CNS). While some immunological events may exacerbate the effects of injury (7), others mitigate it. For example, CNS injury may induce a protective immune response mediated by autoimmune T cells specific for a brain antigen (8, 9). While such cellular autoimmunity has been shown to protect against neuronal loss in traumatic injury, relatively little is known about the role of autoantibodies in brain injury.

Antibodies specific for self antigens, autoantibodies, occur naturally in normal serum. These antibodies play an important role in the removal of dead cells (10–13) by binding to their surface to enhance phagocytosis, a process known as opsonization (14). For example, IgG autoantibodies against keratin filaments promote the phagocytosis of dead keratinocytes (15), while other autoantibodies opsonize senescent or damaged erythrocytes promoting their removal by spleen and liver macrophages (16, 17). Similarly, IgM autoantibodies that bind oligodendrocytes have been isolated, and it has been suggested that they facilitate the opsonization and clearance of these cells by macrophages (18).

Under normal conditions, the blood-brain barrier (BBB) is permeable to some antibodies circulating in the serum (19). In addition, injury to the brain often disrupts the BBB, allowing circulating antibodies as well as other serum proteins greater access to the brain (20–22). As a result, neurons near an injury of the brain are exposed to these circulating antibodies and serum proteins. Indeed, it has been reported that IgG is associated with neurons and glia in the cerebral cortex surrounding a percussive brain injury (23).

The mechanisms mediating neuronal death after injury to the CNS are not fully understood, but substantial evidence indicates a role for free radical generation and nonenzymatic lipid peroxidation (24, 25). The products of lipid peroxidation may modify proteins covalently, and this reaction can alter the immunogenicity of these proteins (26, 27). Furthermore, antibodies that recognize proteins cross-linked to lipid peroxidation products have been found in normal mice and humans (27, 28). These results suggest that lipid peroxidation in dying neurons may lead to the production of antigenic proteins that are capable of being recognized by circulating immunoglobulins. Similarly, the loss of cell membrane integrity by dying neurons accompanied by the exposure of phosphatidyl serine on the outer leaflet of the membrane might be sufficient to trigger an immune response (29). In order to explore the possibility that immunoglobulins recognize...
and bind to dying neurons after an injury of the adult brain, we used immunocytochemical techniques to identify IgG-positive cells in sections from the brains of rats that had survived for various intervals after receiving a unilateral lesion of the visual cortex.

MATERIALS AND METHODS

Surgical Procedures

Twenty-two young adult male Holtzman rats (250–275 g) were used in this study. All animal procedures were performed in accordance with protocols approved by the Institutional Animal Use and Care Committee at the University of Wisconsin–Madison. Nineteen of the rats were anesthetized initially with an intramuscular injection of a mixture of 90 mg/kg ketamine HCl and 9 mg/kg xylazine and maintained under surgical anesthesia with 0.5% halothane. Using aseptic procedures, a bone flap over the right visual cortex was removed and a unilateral lesion of the visual cortex, areas 17, 18, and 18a, was made by subpial aspiration (Fig. 1A). The lesion cavity was filled with a gelatin sponge moistened with 0.9% saline, the bone flap was replaced, and the scalp wound was closed. The animals then recovered from the anesthesia on a heating pad and were returned to their cages. Following postoperative survival periods of 4, 8, and 12 h and 1, 2, 3, 4, 7, and 14 days, the animals were reanesthetized deeply with an i.p. injection of 60 mg/kg pentobarbital sodium and perfused intracardially with 500 ml of a mixture of 4.0% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer. The brains were stored in the fixative at 4°C overnight and then sectioned in the coronal plane at 30 μm using a vibrating blade microtome. Three rats did not receive a cortical lesion and were used as controls. Two of these rats were perfused and postfixed with 4.0% paraformaldehyde, and the other was perfused and postfixed with 4.0% paraformaldehyde and 0.5% glutaraldehyde.

Immunocytochemistry

Sections were washed in 0.125% Triton X-100 in 0.1 M phosphate buffer and blocked for 1 h in 1% normal goat serum in phosphate buffer. Sections then were incubated with one of the following biotinylated antibodies: (a) goat anti-rat IgG, (b) goat anti-rabbit IgG with less than 1% cross-reactivity to rat IgG, (c) goat anti-mouse IgG with 12% cross-reactivity to rat IgG, (d) horse anti-mouse IgG with 50% cross-reactivity to rat IgG, (e) horse anti-mouse IgG with 50% cross-reactivity to rat IgG pre-adsorbed with rat serum, (f) goat IgG (g) goat anti-rat IgM, (h) sheep anti-mouse IgG F(ab′)2, or (i) horse anti-mouse IgG conjugated to Texas Red (Vector Laboratories, Burlingame, CA; 1:50). Antibodies (a) through (f) were obtained from Vector Laboratories and used at a dilution of 1:300; antibodies (g) and (h) were obtained from Sigma Aldrich (St. Louis, MO) and used at a dilution of 1:300. Serum albumin was detected using a rabbit antiserum to rat albumin (ICN Pharmaceuticals, Costa Mesa, CA; 1:250) followed by biotinylated goat anti-rabbit IgG (Vector Laboratories). The sections stained with biotinylated antibodies were incubated in streptavidin-conjugated horseradish peroxidase (Vector Laboratories), which was resolved by using diaminobenzidine as a chromogen.

Counts of IgG-Positive Cells

Animals that survived for 2 days after the cortical lesion were used for counting the number of cells that were immunopositive for IgG. The counts were made in each animal from 3 evenly spaced sections through the cortical lesion with a Whipple eyepiece grid and a 100× oil-immersion objective. At the magnification used, the eyepiece grid subtended 80 μm on each side. It was positioned over the cortex immediately adjacent to the border of the cortical lesion at one end of the lesion, and the grid then was moved in a stepwise fashion until the entire strip of cortex, 320 μm in width, had been traversed (Fig. 1B). Within this strip, all IgG-immunopositive cells that were in the plane of the nucleus were counted. The outline of the nucleus of each cell that was counted was traced with a drawing tube at ×1,000.

The outline of each of the 3 cortical sections used for the cell counts was traced at ×23, and the 320-μm-wide region bordering the lesion in which the cells had been counted was drawn on each tracing. The tracings were digitized, and the area enclosed by the border region on each tracing was computed. Because we defined the border region as a constant distance from the edge of the lesion, the area of this cortical strip varied directly with the mediolateral extent of the lesion. Cell counts for each section were corrected for split-cell error (30) by using the mean nuclear area of the cells counted. The corrected cell counts for each section were divided by the measured area of the border region in which they were counted in order to determine the density of immunostained cells per mm². The cell densities for each of the 3 sections from each brain were averaged to give a mean cell density for each animal. The mean for each animal then was used in computing the mean and standard error of the mean for the group. The mean difference in the number of IgG-immunopositive neurons per mm² in sections stained with an antibody against rat IgG or with an antibody with 12% cross-reactivity to rat IgG was evaluated with a two-tailed t-test. The difference method for Pearson’s r was used to determine the correlation coefficient between the percent reactivity to rat IgG of 3 of the antibodies used in these experiments to the number of immunostained cells observed with each antibody.

RESULTS

At postoperative survival times from 4 h to 7 days, we observed intense immunostaining for IgG in cells located...
near the cortical lesion (Fig. 2). The immunostaining appeared to be specific in that only a small number of the many cells near the cortical lesion were positive for rat IgG (Fig. 3A). Cell somas and processes were stained (Fig. 2A, B), and the majority of immunostained cells displayed cytological features characteristic of neurons. Consistent with an earlier report (31), some of the IgG-positive cells near the lesion resembled astrocytes (Fig. 2C). No immunostained cells were seen in the operated cortex outside of the immediate area surrounding the lesion, in the contralateral cortex, in the thalamus, or in the hippocampus. The immunostaining for rat IgG was most intense and stained the greatest number of neurons 24 h after the lesion. The staining gradually decreased in intensity and cell number until at postoperative day 14 there were no longer any neurons immunostained for rat IgG (Fig. 4A–E).

Glial Cell Activation and Inflammation Adjacent to the Cortical Lesion

Following a CNS lesion and its concomitant disruption of the BBB, cells and molecules of the hematopoietic system, such as leukocytes, macrophages, red blood cells, immunoglobulins, and circulating cytokines, enter the brain (22, 23). In addition, reactive astrogliosis occurs in the vicinity of the lesion as demonstrated by increased GFAP staining of cells near the lesion, but not in unoperated cortex (Fig. 4F–J). Both astroglial cell activation and rat IgG-immunostained neurons occur within the same region of cortex that borders the lesion (Fig. 4).

Effects of Different Fixatives

Three rats did not receive a cortical lesion and served as controls. In sections from the brains of 2 of these animals, which were fixed with 4.0% paraformaldehyde alone, small, scattered patches of cells adjacent to blood vessels in the cortex were stained for IgG in agreement with a previous report (25). The authors of this report hypothesized that such perivascular IgG staining is due to local microvascular permeability to IgG that occurs in the normal brain. A possible explanation for the localized perivascular IgG immunostaining seen in the 2 control animals in the present study, but not in the operated rats, is the omission of 0.5% glutaraldehyde from the fixative used for these 2 controls. Glutaraldehyde strongly cross-links proteins, and its inclusion in a fixative may decrease the antigenicity of some proteins both by altering protein structure and by restricting access of an antibody to its antigen (32). To test the possibility that glutaraldehyde might suppress the IgG-positive perivascular staining, we fixed the brain of a third control rat with 4.0% paraformaldehyde and 0.5% glutaraldehyde, and immunostained sections from this brain for IgG. No perivascular IgG staining was observed, demonstrating that a weak concentration of glutaraldehyde is capable of suppressing the scattered perivascular IgG staining that is seen in the brains of normal animals that have been fixed with paraformaldehyde alone. However, the concentration of glutaraldehyde, 0.5%, that suppresses perivascular IgG staining in normal animals does not suppress IgG staining in...
cells located near a cortical lesion in operated animals. This suggests that the binding of IgG to these cells is sufficiently strong that its immunological detection is preserved in the presence of glutaraldehyde.

Excluding the Involvement of Endogenous Peroxidases

It is well known that endogenous peroxidases can oxidize diaminobenzidine (DAB) independently of the catalytic action provided by horseradish peroxidase-conjugated immunocytochemical reagents. This independent oxidative pathway raises the possibility that the immunostaining we observed may not have resulted from an immunological reaction. To rule out this possibility, we stained sections for IgG after first quenching endogenous peroxidases by incubating the sections in 3% H2O2 for 5 min. This treatment did not eliminate the cellular immunostaining for IgG. In addition, to eliminate DAB histochemistry entirely, we stained sections for IgG with an antibody conjugated to Texas Red. We observed the same cell-specific pattern of IgG staining that was seen in DAB-reacted sections, indicating that the cellular IgG staining is not an artifact of DAB histochemistry, but depends instead upon an immunological reaction (data not shown).

Fc Receptors

Under certain conditions, neurons in the CNS may express Fc receptors (33, 34). To test the possibility that the neurons stained with anti-rat IgG antibody were expressing Fc receptors that bound the Fc region of the biotinylated antibody, we incubated sections with an anti-IgG F(ab')2 antibody that lacks the Fc region. In these sections we also observed neuronal immunostaining for IgG comparable to that described above, indicating that the IgG immunostaining was due to an antigen-antibody reaction between rat IgG and the anti-rat IgG antibody (Fig. 5).

Immunostaining is Specific for Rat IgG

In order to provide evidence against the possibility that the anti-rat IgG antibody was recognizing an epitope other than rat IgG on the immunostained neurons near the lesion, we conducted a number of controls. Initially, we incubated sections with a panel of biotinylated antibodies that varied in their percent cross-reactivities to rat IgG. The staining with each antibody was compared to that produced by a biotinylated antibody raised directly against rat IgG. Counts of the number of immunostained cells near the cortical lesion demonstrated that when the anti-rat IgG antibody was used an average of 26 neurons/mm2 were stained. However, when anti-mouse IgG antibodies with 50% or 12% cross-reactivity to rat IgG were used, 14 neurons/mm2 and 4 neurons/mm2, on average, were stained (Fig. 6). Thus, antibodies with 50% and 12% cross-reactivity to rat IgG stained 53% and 14% of the number of cells, respectively, that were stained by the anti-rat IgG antibody. No immunostaining was seen with an anti-rabbit IgG antibody that had less than 1% cross-reactivity to rat IgG. Similarly, neither an anti-mouse IgG antibody with 50% cross-reactivity to rat IgG that had been pre-adsorbed with rat serum nor an anti-rat IgM antibody produced any staining. Lastly, as a control for nonspecific IgG binding, we incubated sections with biotinylated goat IgG. We did not observe any IgG immunostaining of cells in these sections (Table). Taken together, these results strongly suggest that the specific antigen in these experiments is rat IgG.

Rat IgG Associated with Injured Cells does not Result from Passive Diffusion

Previous studies have reported that IgG and serum albumin can be localized in neurons following ischemia, and it has been proposed that injured cells may act as nonspecific "sinks" for serum proteins if the BBB is disrupted (35, 36). To investigate whether the IgG staining we observed might have arisen from the nonspecific diffusion of serum proteins into injured cells, we immunostained sections from operated animals with an antibody raised against rat albumin. We reasoned that if the observed IgG staining resulted from the nonspecific diffusion of serum proteins into injured cells, then cells near the cortical lesion also should be immunopositive for serum albumin, a protein approximately 26 kD smaller than IgG. However, no cells in the cortex of operated animals were immunoreactive for albumin (Table). To determine if the lack of immunostaining for rat serum albumin resulted from the antibody that was used or protocol that was followed, we stained for albumin in hepatocytes of sections from the liver using the same antibody and following the same protocol that had been used for staining sections of the brain. We observed intense immunostaining for albumin in hepatocytes.

Rat IgG is Associated with Dying Neurons after Disruption of the BBB

As mentioned, cells immunostained for IgG were seen only in the cortex adjacent to the lesion. Many neurons in this area have been axotomized or have had their blood supply interrupted by the cortical lesion and will die. The presence of immunostained neurons with beaded (Fig. 7A) and fragmented processes (Fig. 3) and/or vacuolated cell somas (Fig. 7B, C) indicates that cell death is well underway in many of the neurons that are immunoreactive for IgG. The projection neurons in the lateral geniculate nucleus (LGN) also were axotomized by the cortical lesion and are known to be undergoing cell death at many of the survival times studied (37–40). However, an intact BBB surrounded these dying LGN neurons and none were immunostained for IgG (data not shown).
Fig. 3. Abnormal and beaded processes of rat IgG-immunostained neurons near a lesion of the visual cortex indicate neuronal degeneration. A: A single neuron stained positively for rat IgG (left of asterisk) and is surrounded by many other unstained neurons. B and C: Two examples of rat IgG-immunostained neurons display abnormal processes. Inserts show magnified views of the beaded and fragmented dendrites indicated by arrows. Sections were counterstained with cresyl violet. Scale bar: C = 10 μm (also applies to A and B).

Fig. 4. Time series of rat IgG and GFAP staining in the cortex bordering on a lesion. A–E: No cells stained positively for rat IgG in the unoperated cortex (A). However, at a postoperative survival time of 1 day, many neurons near the cortical lesion stained intensely for rat IgG (B). At 3 days the number of rat IgG stained neurons decreased slightly (C), and at a postoperative survival time of 7 days, both the number of stained neurons and the intensity of staining has decreased further (D). By 14 days following the lesion, rat IgG-immunostained neurons are absent (E). F–J: There was a low level of GFAP immunoreactivity in the unoperated cortex (F). In contrast, GFAP staining was intense in the region near the cortical lesion at a postoperative survival time of 1 day (G) and remained intense at 3, 7, and 14 days after the lesion (H–J). Scale bars: E = 20 μm (also applies to A–D); J = 20 μm (also applies to F–I).

Fig. 5. Immunopositive neurons stained with an anti-IgG F(ab′)2 antibody that lacks the Fc region. A: Two neurons that stain positively for IgG in the vicinity of a cortical lesion at a postoperative survival time of 2 days. B: A single IgG-positive neuron that borders the cortical lesion 2 days postoperatively. IgG staining appears brown. Sections were counterstained with cresyl violet to show all cell somas. Scale bar: B = 10 μm (also applies to A).

DISCUSSION

We present evidence here that an autoimmune recognition of injured and dying cells occurs following a cortical lesion of the brain that incidentally disrupts the BBB. We show that when sections from the damaged brain are incubated with antibodies to IgG, these antibodies bind selectively to neurons located near a cortical lesion and that many of these neurons are in advanced stages of cell death. Neither IgM nor the serum protein albumin is associated with these cells, strongly suggesting the existence of a specific autoimmune reaction between circulating IgG molecules that have entered the injured brain and dying neurons.

Normally, the BBB prevents significant entry of IgG into the brain (22). However, there are important exceptions. For instance, neurons in hypothalamic and brainstem nuclei that send projections to areas without a BBB...
Fig. 6. The number of immunolabeled neurons per mm² near a visual cortex lesion increases with the percent cross-reactivity of the antibody to rat IgG. An antibody with 12% cross-reactivity to rat IgG stained significantly fewer neurons per mm², 3.7 ± 0.7 SEM, than an antibody with 100% cross-reactivity, 25.9 ± 2.6 SEM (p < 0.0002, two-tailed t-test). All data are from rats that survived 48 h after the cortical lesion. The results for the sections stained with an antibody possessing 50% cross-reactivity to rat IgG are based on 1 animal, while the results for the sections stained with antibodies possessing 12% and 100% cross-reactivity are each based on 4 animals. The coefficient of correlation between the number of immunostained cells observed and the reactivity to IgG of the antibodies used is 1.0 (Pearson's r).

Fig. 7. IgG-immunoreactive neurons near a visual cortex lesion that are dying 24 h (A) and 48 h. (B, C) postoperatively. A: Large neuron displays beaded dendrites (arrows) and vacuolated cytoplasm, cytological features characteristic of dying neurons. B and C: Large cytoplasmic vacuoles (arrows) in intensely immunostained neurons indicate an advanced stage of degeneration. Scale bar: C = 10 μm (also applies to A and B).

TABLE
The Presence (+) or Absence (−) of Neuronal Immunostaining Near a Cortical Lesion for Various Antibodies*

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Neuronal Immunostaining</th>
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<tbody>
<tr>
<td>Anti-rat IgG</td>
<td>+</td>
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<tr>
<td>Anti-mouse IgG</td>
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<td>(50% cross-reactivity to rat IgG)</td>
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<tr>
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<td>Anti-rabbit IgG</td>
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<td>(&lt;1% cross-reactivity to rat IgG)</td>
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<tr>
<td>Anti-mouse IgG (pre-adsorbed with rat serum)</td>
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<td>Anti-rat IgM</td>
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<td>Anti-albumin</td>
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<td>Goat IgG</td>
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* Antibody staining was performed on brain sections from rats killed at various times following a cortical lesion as outlined in Materials and Methods.

** Antibody staining was performed on brain sections from rats killed at various times following a cortical lesion as outlined in Materials and Methods.

† Survival times of 4, 8, and 12 hours and 1, 2, 3, 4, and 7 days.

‡ Survival time of 2 days.

§ Survival times of 12 hours and 2 days.

In other systems, the binding of dying cells by autoantibodies is a well-established mechanism for facilitating the clearance of these dying cells. In the circulatory system, IgG autoantibodies bind to the membranes of senescent erythrocytes, and monocytes and macrophages then bind and engulf IgG-coated erythrocytes (16). The membrane protein recognized by circulating autoantibodies is a degradation product of band 3, an anion exchange protein, which is present on a wide variety of cells, including neurons (42). The degradation product of band 3, senescent cell antigen, marks a cell for elimination by promoting the binding of IgG autoantibodies, which in turn leads to phagocytosis of the cell (43). In addition, apoptotic keratinocytes release insoluble keratin filaments...
that can be recognized by IgG autoantibodies. Monocytes then bind and phagocytize these opsonized aggregates (15). In a similar manner, autoantibodies circulating in the blood may enter the region surrounding a brain injury if the BBB is disrupted, and bind to injured cells that are expressing an antigen in response to the lesion. We propose that the IgG antibodies used in the experiments described here recognized circulating rat IgG immunoglobulins that had entered the brain following a cortical lesion and had bound specifically to cells dying as a result of the lesion.

Some investigators have suggested that the appearance of IgG and other serum proteins in ischemic neurons is due to the passive diffusion of these proteins across a damaged plasma membrane (35, 36, 44). Supporting this proposal, neurons occasionally are found in ischemic brains that are associated with endogenous IgG and also with other serum proteins such as albumin (36, 44, 45). Results such as these imply that cell membrane integrity has been compromised, allowing serum proteins to diffuse into ischemic cells.

In contrast to these results, our experiments show that neurons injured by a lesion of the brain are associated only with IgG, and not with serum albumin. Our results are supported by the findings of Schmidt-Kastner et al (46), which are described in a paper that focuses on various methods for immunoglobulin detection. These authors show that neurons and astrocytes can be immunopositive for IgG, but not for serum proteins, in brains that have been injured by a lactate injection or stab wound. Taken together with the present results, this selectivity argues against the passive diffusion of serum proteins into cells injured by a lesion.

Autoantibodies to IgG are Constitutive

In a primary antibody response there is a 2- to 6-day lag in the production of IgG after introduction of an antigen (14, 47). This lag phase is necessary for the presentation of the antigen, the generation of new antibodies to it, and the clonal expansion of B cells. In view of this delay, the presence of IgG immunostaining in our experiments 4 h after a cortical lesion indicates that autoantibodies to IgG are not produced in response to the lesion. Instead, the detection of IgG associated with injured cells within a few hours after the injury demonstrates that, like many other naturally occurring autoantibodies, these IgG autoantibodies are constitutively present in the sera (12, 13). As with damaged erythrocytes and keratinocytes, we suggest that these autoantibodies bind to dying cells in the brain and promote their subsequent phagocytosis. Indeed, microglia are abundant in the brain and possess Fc receptors capable of binding and initiating the phagocytosis of IgG opsonized targets (48).

IgG-Positive Cells Are Undergoing Cell Death

The biochemical and cytological changes that accompany cell death vary with cell type and the initiating circumstances. Therefore, it is not possible to define a series of steps common to all cells that leads to cell death after injury. Moreover, the immunocytochemical methods used in these experiments are not appropriate for identifying many of the hallmarks of cell death, e.g. the activation of intracellular proteases (49). Nevertheless, there are specific cytological changes such as axonal beading and cytoplasmic vacuolization that are generally recognized to indicate that affected cells are degenerating. These cytological features were displayed by many of the neurons in the vicinity of the cortical lesion that were immunostained for IgG.

The appearance of IgG-associated neurons 4 h after a cortical lesion is compatible with what is known about the time course of some of the rapid events involved in cell death. For example, it has been reported that cultured cells from the embryonic rat striatum begin to die within 4 h of exposure to the calcium ionophore A231187, which disrupts intracellular calcium homeostasis (50). Moreover, the activation of membrane phospholipases, lipases, and lipid peroxidation all have been shown to occur within 20 min of cell injury (51, 52).

In the brains that we examined, we observed many IgG-immunostained neurons in various cytological stages of cell death. However, in individual sections, IgG-immunostained neurons were not numerous in comparison with the total number of neurons in the vicinity of the lesion. This observation is not unexpected. After a cortical lesion, cell death occurs asynchronously among the population of affected cells, and individual dying neurons are cleared rapidly from the brain (29). Therefore, while large numbers of cells may die as a result of the lesion, at any given time relatively few dying cells may be evident. Thus it is not surprising that the selective immunostaining of cells for rat IgG that we have reported appears sparse at each of the survival periods that were studied. Taken together, our results strongly suggest that the immunostaining for rat IgG binding that we have observed selectively in cells adjacent to a cortical lesion is related to the injury and death of these cells.

The potential role for lipid peroxidation products in the generation of covalently modified proteins capable of being recognized by autoantibodies already has been mentioned. Similarly, band 3 degradation is induced by oxidation (53), and therefore it is not unlikely that senescent cell antigen is present on injured neurons that would initiate IgG autoantibody binding. A possible alternative mechanism for the recognition of dying cells by autoantibodies is suggested by the study of Denecker et al (54) on the loss of plasma membrane integrity in cultured cells after different death-inducing treatments. In healthy
cells, the plasma membrane maintains aminophospholipid asymmetry by confining phosphatidyl serine to the inner leaflet of the membrane. However, in L929sAhFas cells, a murine fibrosarcoma cell line transfected with the human Fas receptor (55), incubation with anti-Fas antibodies leads within 10 min to a loss of membrane integrity and the presence of phosphatidyl serine on the outer surface of the membrane. The exposure of phosphatidyl serine on the outer leaflet of the membrane of dying cells is thought to play a role in their recognition and engulfment by phagocytes (29). These results raise the interesting possibility that dying cells in the brain that are presenting phosphatidyl serine may be recognized by circulating IgG autoantibodies.

During fetal development the BBB is slow to form and extensive cell death occurs. IgG molecules are present on subplate neurons in the developing rat brain, and it has been suggested that activated macrophages engulf these IgG-presenting neurons (48, 56, 57). Our results strongly suggest that IgG opsonization of dying cells as a prelude to phagocytosis is not specific to the developing brain, but also occurs in the adult brain when dying cells are exposed to serum immunoglobulins.

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REFERENCES


Soares HD, Hicks RR, Smith D, McIntosh TK. Inflammatory leukocyte recruitment and diffuse neuronal degeneration are separate pathological processes resulting from traumatic brain injury. J Neu- rosci 1995;15:8223–33
Abercrombie M. Estimations of nuclear populations from micro- tome sections. Anat Rec 1946;94:239–47
Bernstein JJ, Goldberg WJ. Injury-related spinal cord astrocytes are immunoglobulin-positive (IgM and/or IgG) at different time periods in the regenerative process. Brain Res 1987;426:112–18

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41. Broadwell RD, Brightman MW. Entry of peroxidase into neurons of the central and peripheral nervous systems from extracerebral and cerebral blood. J Comp Neurol 1976;166:257–83
42. Cover CC, Poulin JE, Gustafson MR, Wyant T, Gamble DN, Kay MM. Posttranslational changes in band 3 in adult and aging brain following treatment with ergolide mesylates, comparison to changes observed in Alzheimer’s disease. Life Sci 1996;58:655–64
49. Ling C, Kalil RE. The cleavage of actin by caspase-3 in axotomized neurons in the lateral geniculate nucleus (LGN) of the adult rat. Soc Neurosci Abst 1999;25:759

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